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Cold-shock induced genes in *Yersinia enterocolitica*: Processing of Csp mRNA and transcriptional regulation of DEAD box RNA helicase

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“Arbeite, als ob Du erwartest, hundert Jahre zu leben, und lebe, als ob Du erwartest, morgen zu sterben!”

Serbisches Sprichwort

“Radi kao da ćeš sto godina da živiš i živi kao da ćeš sutra da umreš!”

Srpska poslovica

It takes a minute to find a special person, an hour to appreciate them, a day to love them, but then an entire life to forget them.

Friend 

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

DNA	desoxyribonucleic acid
RNA	ribonucleic acid
ds	double stranded
nt	nucleotides
bp	base pairs
kb	kilobase pairs
ORF	open reading frame
UTR	upstream region
aa	amino acid(s)
C-terminal	carboxy-terminal
N-terminal	amino-terminal
Csp	Cold-shock protein
CSC box	Cold-shock Cut box
PNPase	polynucleotide phosphorylase
RNase	ribonuclease
LPS	lipopolysaccharide
Yops	Yersinia outer proteins
DEAD box	(Asp (D) – Glu (E) – Ala (A) – Asp (D)) motif
TPR	tetratricopeptide repeat domain
EGFP	Enhanced Green Fluorescent Protein
RNP	RNA binding protein
nmRNA	non-messenger RNA
siRNA	small interfering RNA
miRNA	micro RNA
RNAi	RNA interference
EDTA	Ethylenediaminetetraacetic acid
SDS	sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane
× *g*	relative centrifugal force

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SUMMARY

The psychrotolerant *Y. enterocolitica* is an emerging foodborne pathogen and has the ability to proliferate at low temperatures (down to –5°C), while *E. coli* has a minimum of temperature growth at 8°C. This characteristic of *Y. enterocolitica* is important for bacterial growth in contaminated refrigerated food, water and blood supply. Answering the question, how growth at low temperature could be inhibited, requires fundamental knowledge about specific metabolic factors which enable this organism to accommodate rapidly and to multiply at low temperatures.

Temperature downshift from 30°C to 10°C leads to the induction of mRNA transcription of the cold-shock protein tandem gene *cspA1/A2* (Neuhaus, Francis et al. 1999). A number of *cspA1/A2* specific degradation intermediates accumulate at the end of the cold acclimation period. Primer extension assays demonstrated that these degradation products had 5´ terminus at a common core sequence 5´-AGUAAA-3´ (Neuhaus 2000). In this study we wanted to test if this sequence was responsible for cleavage and degradation of *cspA1/A2* mRNA. The AGUAAA core sequence was found to be present in the *csp* genes of many Gram negative and Gram positive bacteria. Cleavage was also obtained in the mRNA transcribed from the *E. coli cspB* gene, which contains two of these core sequences. After inserting this motif into a plasmid-bound *lacZ* gene in *Y. enterocolitica*, the mRNA of this construct was also cleaved within the motif. Moreover, changing the motif from AGUAAA to AGUCCC dramatically reduced cleavage, but did not completely abolish it. RNase E dependent cleavage was shown for the construct with AGUAAA motif according to the *in vivo* analysis of *E. coli* mutants. *In vitro* cleavage of total mRNA and synthetic oligoribonucleotides carrying this motif by the catalytic domain of RNase E and RNase G provided evidence that the AGUAAA sequence played a role in allowing *cspA1/A2* transcripts to be efficiently cleaved by RNase E and not by RNase G. Since an excess of *cspA* mRNA block ribosomes, efficient removal of this mRNA species from the cell is essential for later stages of cold adaptation.

Further it was interesting to analyze other factors that may also contribute to the mRNA degradation at low temperatures. During cold-shock conditions PNPase has been considered to play an important role in selective degradation of *csp* mRNAs and it was shown that *pnp* gene is also cold-shock inducible (Goverde, Huis in't Veld

et al. 1998). We have characterized *nlp1* and *deaD* genes downstream from *pnp* gene in *Y. enterocolitica* and have shown that they are induced in response to temperature downshift during exponential growth phase. By Northern hybridization it was shown that *pnp*, *nlp1* and *deaD* are part of a polycistronic operon and they are cotranscribed as cold temperature induced mRNA. Stabilization of mRNA is involved in the synthesis of DeaD RNA helicase, due to the accumulation of polycistronic transcript during a cold shock. Two regions in front of *pnp* gene and in front of *deaD* gene were analyzed for promoter activity by primer extension and by transcriptional *egfp* fusion constructs. In addition to the *pnp* promoter, primer extension experiments revealed the existence of two further transcription start sites upstream of the *deaD;* P*deaD1* and P*deaD2*. Analysis of transcriptional *egfp* fusions showed that transcription from the P*pnp* promoter was constitutive at 30°C and 10°C and transcription from the two *deaD* promoters was enhanced 4 hours after cold-shock with a further increase of the P*deaD2* transcriptional activity up to 20 hours. At 30°C activity of the P*pnp* and P*deaD2* promoters was lower than the activity of the P*lacZ* promoter which has been used as a control. After cold-shock EGFP activity from the P*pnp* and P*deaD2* promoters was detected after 20h incubation at 10°C, while efficiency of the P*lacZ* promoter was strongly reduced. Results from this work clearly demonstrate that regulation of *deaD* gene expression at low temperature is under transcriptional and posttranscriptional control, and that the identified cold-shock elements contribute to the differential expression of PNPase, Nlp1 and DEAD RNA helicase of *Y. enterocolitica*.

ZUSAMMENFASSUNG

Das psychrotolerante Bakterium *Yersinia enterocolitica* ist ein Lebensmittelkeim, der bei niedrigen Temperaturen bis –5°C zum Wachstum befähigt ist, während *E. coli* bereits bei 8°C ein Wachstumsminimum aufweist. Dieses Charakteristikum von *Y. enterocolitica* ist insofern von großer Bedeutung, als dieser pathogene Keim sich dadurch auch in gekühlten Nahrungsmitteln, in Trinkwasser oder in Blutkonserven vermehren kann. Um die Frage zu beantworten, wie sich das Wachstum von Yersinien bei niedrigen Temperaturen möglicherweise hemmen lässt, ist eine genaue Kenntnis der intrazellulären Vorgänge bei der Kälteadaptation erforderlich.

Nach einer plötzlichen Temperaturerniedrigung („Kälteschock“) von 30°C auf 10°C wird in *Y. enterocolitica* effizient ein bicistronisches Gentandem mit den Hauptkälteschockproteinen CspA1 und CspA2 induziert. Zur schnellen Anpassung an diese neuen Umweltbedingungen gehört nun nicht nur die Induktion neuer mRNAs für Stressproteine, sondern auch deren Regulation auf posttranskriptioneller Ebene durch mRNA-Abbau. Nach erfolgter Anpassung an den Kälteschock mit Hilfe der Kälteschockproteine CspA1/A2 muss zwischenzeitlich in großen Mengen synthetisierte mRNA wieder abgebaut werden, da sonst die Ribosomen von diesem Überschuss an mRNA blockiert werden. Im Anschluss an eine Phase der Kälteanpassung akkumulieren eine Reihe von *cspA1/cspA2*-spezifischen mRNA-Intermediaten, denen die Sequenz AGUAAA am 5´-Ende gemeinsam ist. Diese Konsensussequenz findet sich in den *csp*-Genen vieler Gram-positiver und Gram-negativer Bakterien. Folgerichtig wurden auch in *E. coli* entsprechende Spaltprodukte der mRNA des *cspB*-Genes beobachtet, die zwei AGUAAA-Sequenzen aufweist. In der vorliegenden Arbeit wurde die Rolle der AGUAAA-Sequenz bei der Kälteadaptation von *Y. enterocolitica* untersucht. Dazu wurde die Konsensussequenz zunächst in ein *lacZ*-Gen auf einem pBlueScript-Vektor kloniert. Wie erwartet erfolgte ein Abbau dieser chimären mRNA bevorzugt an der eingefügten Schnittstelle AGUAAA. Eine leicht veränderte Schnittsequenz (AGUCCC) zeigte hingegen einen deutlichen Abfall der Schnittintensität. Der Nachweis, dass dieses Spaltung RNase E-abhängig ist, wurde für Konstrukte mit AGUAAA-Motiv durch entsprechende *in vivo*-Analyse in *E. coli*-Mutanten gezeigt. *In*

vitro Spaltungsexperimente von Gesamt-RNA und synthetischen Oligoribonucleotiden, die dieses Motiv tragen, in Gegenwart der RNase E und der RNase G lieferte den Beweis, dass der Abbau von *cspA1/A2*-mRNA durch die RNase E und nicht durch die RNase G erfolgt, und dass die katalytische Domäne der RNase E die AGUAAA Sequenz erkennt.

Weiterhin wurden zusätzliche Faktoren analysiert, die ebenfalls zum mRNA-Abbau bei niedrigen Temperaturen beitragen. Während der Kälteschock-Phase wird die Induktion der PNPase beobachtet, die eine wichtige Rolle in der mRNA-Degradation von *cspA* spielt (Goverde, Huis in't Veld et al. 1998). In dieser Arbeit wurde ein 6253 bp langes DNA-Fragment aus *Y. enterocolitica* sequenziert, das neben dem *pnp*-Gen auch das *nlp1*- und *deaD*-Gen trägt. Hier wurde gezeigt, dass diese Gene nach einem Kälteschock während der exponentiellen Wachstumsphase induziert werden. Dabei ergab sich, dass die Stabilisierung der nach einem Kälteschock akkumulierten mRNA bei der Synthese der DeaD RNA-Helicase wichtig ist. Durch Northern-Hybridisierung wurde für die Gene *pnp, nlp1* und *deaD* nachgewiesen, dass sie Teil eines polycistronisches, kälteinduzierten Operons sind. Zwei Regionen vor dem *pnp*-Gen bzw. dem *deaD*-Gen wurden mittels Primer-Extension und transkriptioneller *egfp*-Fusionen analysiert. Die Untersuchung dieser 5‘-Regionen bestätigte bzw. deckte den Transkriptionsstart des *pnp*-bzw. *deaD*-Genes auf. Darüberhinaus konnte eine Reihe von DNA-und RNA-Elementen, die charakteristisch sind für kälteinduzierte Gene, gefunden werden; dazu zählen eine 5´-UTR-Region, eine „downstream-box“ sowie eine „cold-shock-box“. Die Analyse der transkriptionellen *egfp*-Fusion zeigte, dass das *deaD*-Gen vom *pnp*-Promotor und von seinem eigenen Promotor während der Kälteschock synthetisiert wird. Die Aktivität des *pnp*- und des *deaD2*-Promotors war bei 30°C niedriger als die Aktivität des *lacZ*-Promotor, der als Kontrolle diente. Die Aktivität des *pnp* Promotors bei 30°C und 10°C bleibt konstant. Der *deaD2*-Promotor offenbar bei 10°C stärker induziert wird als bei 30°C, während die Aktivität des *lacZ*-Promotor bei 10°C stark reduziert wurde. Die hier vorgestellten Resultate zeigen, dass die Regulation der *deaD* Genexpression bei niedrigen Temperaturen unter transkriptioneller und posttranskriptioneller Kontrolle steht.

1 INTRODUCTION

1. 1 Yersinia

Yersiniae, as member of family *Enterobacteriaceae,* are oxidase-negative, gram-negative, rod shaped facultative anaerobes which ferment glucose. The genus *Yersinia* comprises 11 species and includes three human pathogens: *Y. pestis*, the causative agent of bubonic and pneumonic plague (the black death), *Y. pseudotuberculosis*, an intestinal pathogen of rodents which occasionally infects humans, and *Y. enterocolitica*, a common intestinal pathogen of humans. *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* share a number of essential virulence determinants, which enable them to overcome nonspecific immune defense of their hosts. Analogs of these virulence determinants occur in several other bacterial pathogens, including *Salmonella* and *Shigella* species, providing evidence for horizontal transfer of the genetic information for virulence determinants between enteric pathogens (Robins-Browne 1997).

1.1.1 Taxonomy

After several unsuccessful attempts to allocate it to a suitable taxonomic position, *Y. enterocolitica* was finally assigned to the family *Enterobacteriaceae*. *Y. enterocolitica* is highly heterogeneous, being divisible into a large number of subgroups, according to biochemical activity and lipopolysaccharide (LPS) O antigens (Tab. 1.1).

Biotyping is based on the ability of *Y. enterocolitica* to metabolize selected organic substrates. Pathogenic strains, of human and domestic animals, are grouped in biovars 1B, 2, 3, 4, and 5. Not all isolates of *Y. enterocolitica* obtained from soil, water or unprocessed food can be assigned to a biovar, while the vast majority of strains recovered from environmental sources were either non-typeable or were serotypes which have never been implicated in human infections (Aleksic and Bockemuhl 1990). These strains invariably lack the characteristic virulence determinants of the primary pathogenic *Yersinia* sp.

Serotyping of *Y. enterocolitica* is based on LPS surface somatic (O) antigens and flagellar (H) antigens what provides a useful additional tool to subdivide this species in a way that relates to pathologic significance. The situation is further

complicated by the fact that H-antigens are species-specific, while the O-antigens are not. In addition, certain geographical distribution was also present for distinct bio/serotypes. Group of strains (the biotype 1B strains), were mainly isolated in the USA. On the other hand, serotypes O:3 and O:9, responsible for yersinia outbreaks in Europe and Japan were virtually unknown in America (Robins-Browne 1997).

Table 1.1 Relationship between O serovar and pathogenicity of *Y. enterocolitica* and related species

Species	Serovar(s)
Y. enterocolitica	
Biovar 1A	O:4; O:5; O:6,30; O:6,31; O:7,8; O:7,13; O:10; O:14; O:16; O:21; O:22; O:25; O:37; O:41,42; O:46; O:47; O:57
Biovar 1B	**O:4,32; O:8; O:13a,13b**; O:16; **O:18; O:20; O:21**; O:25; O:41,42
Biovar 2	**O:5,27; O:9**; O:27
Biovar 3	**O:1,2,3; O:3; O:5,27**
Biovar 4	**O:3**
Biovar 5	**O:2,3**
Y. bercovieri	O:8; O:10; O:58,16
Y. frederiksenii	O:3; O:16; O:35; O:38; O:44
Y. intermedia	O:17; O:21,46; O:35; O:37; O:40; O:48; O:52; O:55
Y. kristensenii	O:11; O:12,25; O:12,26; O:16; O:16,29; O:28,50; O:46; O:52; O:59; O:61
Y. mollaretti	O:3; O:6,30; O:7,13; O:59; O:62,22;

*Serogroups that include strains considered to be primary pathogens are in boldface.

1.1.2 Virulence

Expression of virulence is tightly regulated at a genetic level. However, the contradictory actions of these determinants probably reflect the ability of Yersiniae to respond to the changing environments they encounter in soil, water, food and host tissues. The virulence determinants of *Y. enterocolotica* are classified into those, which are chromosomally encoded, and those specified by a 70kb virulence plasmid. *Y. enteroclitica* requires a well-defined 70kb virulence plasmid (pYV) for

full virulence, but early studies showed that while pYV is necessary, it is not sufficient to cause disease.

Expression of virulence determinants in *Y. enterocolitica* is very dependent on temperature. Synthesis of specific O-antigen by *Y. enterocolitica* is specified by chromosomal *rfb* locus and is regulated by temperature such that colonies are smooth when grown at temperatures below 30°C but rough when grown at 37°C. All *Y. enterocolitica* strains belonging to the virulent serovars are able to produce outer membrane protein termed invasin encoded by *inv* gene. Invasin plays a key role in the initial penetration of the intestinal epithelium by *Y. enterocolitica* and it was shown that expression of invasin is reduced at temperatures above 30°C. Unlike *inv*, the *ail* gene (attachment-invasion chromosomal locus) is expressed *in vitro* at host temperatures (37°C). Virulence plasmid pYV encodes proteins referred as Yops. When the bacteria make contact with host cells in lymphoid tissue, they are stimulated to synthesize and release Yops which further frustrate the efforts of phagocytes to ingest and remove them. All Yops are produced *in vitro* at 37°C, but not at temperatures below 30°C (Robins-Browne 1997).

1.1.3 Human yersiniosis

Y. enterocolitica is a gram-negative bacterium that emerged in the past two decades as an important enteric pathogen associated with a wide spectrum of clinical manifestations. Infection due to *Y. enterocolitica* was first observed in 1933 in New York. The most common infection is enterocolitis with its diarrheal manifestations. Septicemia with *Y. enterocolitica* has been increasingly recognized in recent years, whereas endocarditis due to *Y. enterocolitica* is a rare manifestation. Yersiniosis may also give rise to a variety of suppurative and autoimmune complications.

1.1.4 Incidence in food

Yersinia enterocolitica is a versatile foodborne pathogen with a remarkable ability to adapt to a wide range of environments within and outside the host. The bacteria typically access their hosts via food or water in which they have grown to stationary phase at ambient temperature. *Y. enterocolitica* is unusual among pathogenic enterobacteria in being psychrotolerant, as evidenced by its ability to replicate at temperatures between 0°C and 44°C. The development of the industrialised food production and increased use of refrigeration for food long

conservation, have greatly enhanced the importance of psychrotolerant bacteria. Extended refrigerated storage of raw and underprocessed food of animal origin, as well as cross-contamination of other food, has all played a role in outbreaks of yersiniosis. Refrigeration has not been restricted to food. Preservation of blood products for transfusion may also have a risk for bacterial contamination. Among the microorganisms involved in transfusion-associated bacteremia, *Y. enterocolitica* and *Pseudomonas fluorescens* have emerged to play an important role, undoubtedly favored by their ability to grow at the common blood storage temperature of 4°C (Tipple, Bland et al. 1990).

1. 2 Adaptation to Low Temperature

Among the various environmental factors that permit the viability, growth and physiology of microorganisms, temperature is of particular interest since it affects immediately the interior of the cells. According to their ability to grow at high, intermediate or low temperatures, microorganisms have been divided into three broad categories: thermophiles, mesophiles and psychrophiles, respectively. The last category has been further subdivided into psychrophiles, which have optimal growth temperatures below 15°C and an upper limit of 20°C, and psychrotrophs (psychrotolerants) which are able to divide at 0°C or below and grow optimally at temperature around 20-25°C (Morita 1975). Psychrophilic and psychrotolerant microorganisms are of particular importance in global ecology since the majority of terrestrial and aquatic ecosystems of our planet are permanently or seasonally submitted to cold temperatures. Microorganisms capable of coping with low temperatures are widespread in these natural environments where they often represent the dominant flora and they should therefore be regarded as the most successful colonisers of our planet (Russell 1990).

Most of our knowledge on how cells respond to rapid temperature downshifts originates from studies on mesophilic microorganisms such as *E. coli* and *B. subtilis*. (Jones and Inouye 1996; Jones and Inouye 1996; Graumann, P. and Marahiel 1996; Thieringer, Jones et al. 1998). In mesophilic bacteria like *E. coli,* cold-shock causes a transient inhibition of synthesis of most cellular proteins, resulting in a growth *lag* period called the acclimation phase. During this acclimation phase, at least 15 different cold-shock proteins are significantly induced, and some of them are essential for cell growth at low temperature (Etchegaray, Jones et al. 1996). That

means that cold-shock result in the transient inhibition of the synthesis of the bulk proteins, the so-called housekeeping proteins. In *B. subtilis*, this inhibition is only partial and at least 75 proteins are permanently synthesised upon cold-shock (Graumann, P., Schröder et al. 1996). The growth of *B. subtilis* continues at a reduced doubling time without apparent growth lag. After cold-shock of *Y. enterocolitica* to 10°C, exponential growth resumes after approximately 80min (Neuhaus, Rapposch et al. 2000), while in *E. coli* the growth is arrested until 240min (Jones, VanBogelen et al. 1987). The response of *E. coli* is somewhat different since the transient inhibition of housekeeping proteins is total and results in a growth *lag* period (Thieringer, Jones et al. 1998). One of the most significant differences between mesophiles and cold-adapted bacteria is that the relative rate of synthesis of most cytosolic proteins is maintained after cold shock. It can be assumed that, as opposed to mesophiles, regulatory factor(s) exist in cold-adapted bacteria prior to cold-shock which allows the maintenance of transitional machinery at low temperature.

Within the proteins that are highly induced after cold shock, there is a family of structurally related proteins, CspA, CspB and CspG. Among them, CspA has been identified as a major cold-shock protein. Major cold-shock proteins from different bacterial species are characterized by the ability to preferentially bind to single-stranded nucleic acid sequences. In both, prokaryotic and eukaryotic organisms this ability has been shown to be due to two RNA-binding motifs RNP-1 and RNP-2 (Graumann, P. and Marahiel 1994; Wolffe 1994). Although a number of roles has been proposed for CSPs, they most likely function as molecular chaperones unfolding mRNA secondary structures formed at low temperatures (Graumann, P., Wendrich et al. 1997; Jiang, Hou et al. 1997; Jones and Inouye 1994).

E. coli and *Y. enterocolitica* are highly related bacteria (Holt 1994). However, the temperature growth ranges of these two organisms are quite different. *Y. enterocolitica* is able to grow at much lower temperatures than *E. coli,* and why these two bacteria differ so greatly in their ability to grow at low temperature is not known.

The question, which arises, is what are the cold-shock specific adaptive mechanisms that allow psychrotolerants to cope with lower temperature better than mesophiles. Although the cold-shock response in psychrotolerant bacteria is still poorly documented, some data indicate similarities but also differences that may

provide a partial answer to this question. Presence of the bicistronic mRNA of *cspA1/A2* can be one reason why *Y. enterocolitica* has better growing at low temperatures (Neuhaus, Francis et al. 1999).

1. 3 mRNA stability and mRNA degradation are important for cold-shock adaptation

It has been discussed, in *E. coli*, that extreme temperature instability of *csp*A mRNA is partially due to its degradation by RNase E (Fang, Jiang et al. 1997), an enzyme that, together with PNPase, forms part of the degradosome complex (Py, Higgins et al. 1996; Blum, Py et al. 1997).

Many RNA molecules undergo specific cleavage reactions in order to attain their mature, functional forms. In addition, each RNA is ultimately degraded to mononucleotides. The processing and degradation of mRNA by the concerted action of endoribonucleases and exoribonucleases are important events in the control of gene expression in all living cells (Belasco and Higgins 1988). Essentially all stable RNA precursors undergo one or more maturation reactions. For example, ribosomal RNA maturation in bacteria is initiated by endonucleolytic cleavage of the primary transcript during its synthesis. These cleavage events release the precursors to 16S, 23S and 5S rRNAs as well as several tRNAs, which are matured by additional endonucleolytic and 3‘-5‘ exonucleolytic cleavages (Srivastava and Schlessinger 1990). Bacterial mRNA maturation reactions are less common (Nicholson 1999). A major pathway for bacterial mRNA decay involves a multicomponent particle, termed the degradosome.

Extensive biochemical and immunological experiments have demonstrated that the RNase E protein is a major protein in degradosome. RNase E is very large protein (1061 amino acids), composed of three distinct domains (catalytic, arginin-rich RNA binding site (ARRBS) and scaffolding region) (McDowall and Cohen 1996). Py *et al.,* purified from *E. coli* a 500 kDa degradosome complex, which contains RNase E, PNPase, the DEAD-box RhlB RNA helicase, enolase, and other unidentified proteins (Py, Causton et al. 1994; Miczak, Kaberdin et al. 1996). Analysis of various truncated RNase E proteins have established that the carboxy-terminus (amino acids 734-1045) serves as the scaffold for the association of PNPase, RhlB helicase and enolase (Vanzo, Li et al. 1998). The N-terminal region

of RNase E is responsible for catalytic activity of RNase E enzyme and seems to be conserved in a variety of bacterial species (Kaberdin, Miczak et al. 1998).

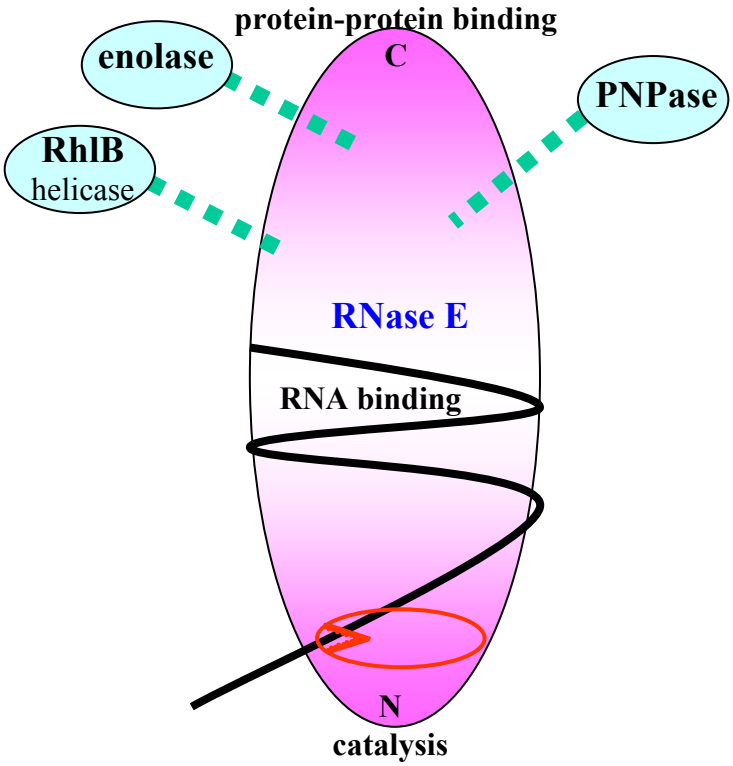


Fig. 1.1 The prokaryotic degradosome. This scheme presents a model for the structural organization of the degradosome (Rauhut and Klug 1999). N-terminus is responsible for catalysis and C-terminus is responsible mainly for protein-protein binding activity of RNase E enzyme which has been assumed to be the main scaffold for degradosome complex. The model reflects the current ideas about the interaction of known degradosome components, whereas PNPase is polynucleotide kinase encoded by *pnp* gene.

The RNA cleaving activity of RNase E has been studied with a variety of standard substrates such as the 9S rRNA precursor of 5S rRNA, the mRNAs encoding OmpA, TrxA, and ribosomal proteins S20 and S15 (*rps*T and *rps*O). Cleavage products contain 5‘ phosphates and 3‘ hydroxyl groups, the cleavage preferentially occurs in single-stranded A/U-rich regions (Mackie 1992). The original proposals describing the structural constraints of an RNase E cleavage site in *E. coli* required a single-stranded consensus sequence (A/G)AUU(A/U) and additional

lateral stabilizing stem-loop anchors (Ehretsmann, Carpousis et al. 1992; Cormack and Mackie 1992; Rauhut and Klug 1999).

The role of secondary structures adjacent to cleavage sites has been debated extensively. Most cleavage sites are preceded or followed by a stable stem-loop structure. Probably stem-loops do not function as direct recognition motifs, but rather serve to stabilize the local secondary structure, so that the adjacent cleavage site remains single stranded. (Coburn and Mackie 1999).

Additionally, Braun et al. (1998) have shown that ribosomes inhibit RNase E cleavage, 10 nucleotides downstream from the UAA codon of the *rps*O coding sequence in *E. coli*. Ribosomes are responsible for triggering the exonucleolytic decay of the message mediated by polynucleotide phosphorylase. Jurgen, et al. (1998), have reported that the stability of *gsi*B mRNA from *B.subtilis* is enhanced by a strong ribosome binding site (RBS). A similar mechanism could occur within the *cspA1/A2* mRNA from *Y. enterocolitica*. It has been shown that the *cspA1/A2* gene duplication in *Y. enterocolitica* is cold inducible, and that both monocistronic and bicistronic templates are transcribed from this gene (Neuhaus, Francis et al. 1999). Transcripts *cspA1/A2* are rapidly induced upon cold-shock and then degrade rapidly upon subsequent cold adaptation of the cell. This could imply that the cellular concentration of *cspA1/A2* mRNA must be carefully maintained.

1. 4 Function of small, non-messenger RNAs

The expression of the cold inducible genes is controlled at the levels of transcription, translation and protein stability. The protein amount, for example CspA, Pnp, and CsdA, should be well adjusted in response to environmental signals (Brandi, Spurio et al. 1999; Wang, Yamanaka et al. 1999; Zangrossi, Briani et al. 2000). How these changes in the environment are sensed and how different signals are integrated is not well understood.

Recently it was shown that the small untranslated regulatory RNA DsrA is necessary for the low temperature expression of the general stress sigma factor RpoS (Repoila and Gottesman 2001). The requirement of small, *trans*-acting RNAs such as DsrA to stimulate RpoS translation allows the sensing of a variety of physiological conditions to result in major changes in the cell's capacity to react to sterss. Changes in synthesis of DsrA with environmental variations such as

temperature will afect RpoS translation as well as other targets of DsrA (Repoila and Gottesman 2001). DsrA is only one of at least three small RNAs affecting RpoS translation. The other two small RNAs, RprA and OxyS, are synthesized in response to other signals (Altuvia, Weinstein-Fischer et al. 1997; Zhang, Altuvia et al. 1997; Majdalani, Chen et al. 2001). Multifunctional RNAs such as DsrA are likely to exist in all organisms. They allow integration of multiple environmental signals to cordinatelly regulate multiple outputs. Such coordination can be added independently operating transcriptional and posttranscriptional controls, providing even more responsiveness for the cell.

Cells from all known organisms contain two different kinds of RNAs: mRNAs, which are translated into proteins and non-messenger RNAs, also referred to as non-coding RNAs, which are not translated into proteins. Non-messenger RNAs (nmRNAs) range from very large, for example rRNAs, to extremely small (21-23 nt) micro RNAs (miRNAs) (Huttenhofer, Brosius et al. 2002). Small nmRNAs are present in many different organisms, ranging from bacteria to mammals. These RNAs carry out a variety of biological functions. Many of them acts as regulators of gene expression at posttranscriptional level, either by acting as antisense RNAs, by binding to complementary sequences of target transcripts, or by interacting with proteins. Regulatory RNAs are involved in the control of a large variety of processes such as plasmid replication, phage development, viral replication, bacterial virulence and global circuits in bacteria in response to environmental changes (Argaman, Hershberg et al. 2001). Quite recently, two novel classes of small nmRNAs were discovered: miRNAs and small interfering RNAs (siRNAs), both of 21-23nt length (Ruvkun 2001) which can be most readily distinguished from each other by the unique aspects of their biogenesis (Fig. 1.2). siRNAs are cleaved from very long precursor double-stranded RNA (dsRNA) by the enzyme Dicer, which is related to RNase III (Huttenhofer, Brosius et al. 2002). miRNAs are also processed by Dicer but they arise from stem-loop structures of much shorter size (Huttenhofer, Brosius et al. 2002). Subsequently, siRNAs and miRNAs species are assembled into a multi-component protein complex that guides the sequence-specific recognition of the target mRNA (Fig. 1.2). This complex is referred to as the RNA–induced silencing complex (RISC) (Mourelatos, Dostie et al. 2002). It is accepted that siRNAs function by binding RISC (containing the siRNA) to mRNA followed by cleavage of the mRNA target. It was shown that cleavage occurs

exactly in the center of the short double-stranded siRNA/mRNA complex (Elbashir, Lendeckel et al. 2001). Activated RISC complexes can regulate gene expression at many levels. Almost certainly, such complexes act by promoting RNA degradation and inhibition of translation (Fig. 1.2). This silencing mechanism is characterized in eukaryotes. There is one report that describes that parallel complementary RNA can function to inhibit gene expression in *E. coli* (Tchurikov, Chistyakova et al. 2000). It was found that adaptation to cold-shock is blocked when the 143bp sequence of the 5' UTR region of the *cspA* mRNA is overproduced (Jiang, Fang et al. 1996). However there is no further experimental proof provided about existence of silencing mechanisms in prokaryotes. To have a contribution to the silencing mechanism in prokaryotes further studying of cold-shock response and cold-shock adaptation in bacteria can be important.

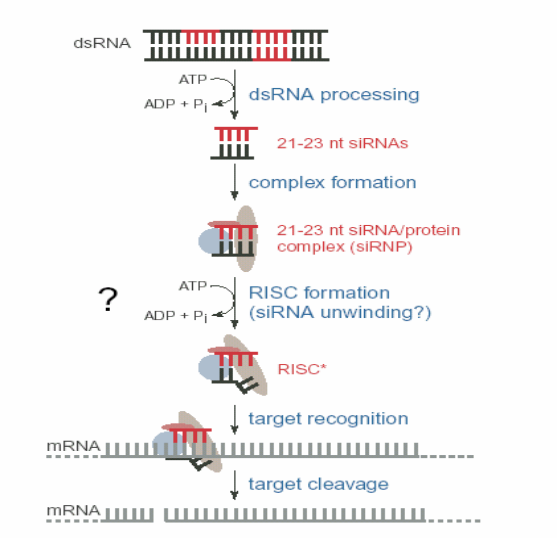


Fig. 1.2 Model for the mechanism of action of siRNAs (RNAi) (Zamore 2001). Silencing triggers in the form of double stranded RNA may be presented in the cell as synthetic RNAs, replicating viruses or may be transcribed from specific genes. These are recognized and processed into small interfering RNAs (siRNAs) by Dicer. The duplex siRNAs are passed to RISC (RNA – induced silencing complex), and the complex becomes activated by unwinding of the duplex. Different DEAD box RNA helicases can make unwinding of the duplex by binding of ATP. Activated RISC can further regulate gene expression by specific RNA degradation or translational inhibition.

The function of miRNAs and siRNAs is presently unknown, but the discovery of the ribonucleoproteins (RNPs) such as degradosome complex and RISC

complex is of considerable significance. RNAs function in cells in the form of RNPs, and the identification of their components is essential for understanding the function of the RNAs with which they are associated.

The 500 kDa RISC protein complex has been shown to assemble with siRNAs that subsequently recognize homologous mRNAs and guide their cleavage by an unidentified nuclease(s) present in RISC (Hammond, Bernstein et al. 2000). Discovery of DEAD box putative RNA helicase in RNPs, indicates that it mediate miRNAs unwinding or RNP restructuring events during the maturation of RNAs and/or in downstream events such as target RNA recognition (Mourelatos, Dostie et al. 2002). Different RNA helicases can associate with RNPs and provide different properties of RNPs in gene expression and regulation processes. Therefore, it would be important to characterize a specific DEAD box RNA helicases that could be involved in mRNA degradation (RhlB helicase) or in cold-shock adaptation (DeaD helicase) in *Y. enterocolitica*.

1. 5 Involvement of DEAD box RNA helicase in cold-shock adaptation

A range of mechanisms providing survival for mesophilic bacteria have been identified by examining the cold-shock response (reviewed by Thieringer, Jones et al. 1998; Phadtare, Alsina et al. 1999). Regulation of cold-schock adaptation in *E. coli* occurs at numerous levels, including transcription, mRNA stabilization, translation, and has been best characterized for the most abundant CSP gene, *cspA*. At the transcriptional level, *cspA* is regulated positively by an AT-rich upstream element, and negatively by a cold box (Jiang, Fang et al. 1996; Mitta, Fang et al. 1997). mRNA stabilization also plays a significant role in *cspA* transcript accumulation (Brandi, Pietroni et al. 1996; Goldenberg, D., Azar I, Oppenheim AB 1996). Although the mechanism regulating CSP expression is not known, the rate-limiting step under cold-shock conditions is the initiation of translation (Jones and Inouye 1994). A cold-shock ribosome adaptation model which allows this rate-limiting step to be overcome by the association of three CSPs, translation initiation factor 2, CsdA (RNA helix destabilization), and RbfA (ribosome binding factor A), with the ribosome, converting it into a cold-resistant translatable state, has been proposed (Thieringer, Jones et al. 1998). In this scenario, CsdA, is an RNA helicase

which possesses RNA helix-destabilizing activity, and proposed to remove secondary structures in the highly structured upstream region (UTR) of *E. coli csp*A mRNA, thereby facilitating translation initiation (Jones, Mitta et al. 1996).

The majority of RNA helicases are DEAD-box proteins, so named for the largely conserved “DEAD" (Asp-Glu-Ala-Asp) motif. DEAD box proteins are found in a wide range of organisms, ranging from bacteria to mammals, and are involved in a variety of processes such as ribosome assembly, initiation of translation, mRNA degradation, cell growth and differentiation (Nicol and Fuller-Pace 1995; Nicol, Causevic et al. 2000). Although sequences encoding putative RNA helicases have been identified in a number of prokaryotic species, including those of the genus *Bacillus* and *Archaea,* they have been studied most extensively in *E. coli*, whose genome encodes five different DEAD box protein genes (*rhlB, srmB, dbpA, csdA* and *rhlE*). Functions that have been identified for *E. coli* DEAD box RNA helicases include importance in the mRNA degradation process - *rhlB* (Py, Higgins et al. 1996), ribosome biogenesis - *srmB* (Nishi, Morel-Deville et al. 1988), specifically stimulated ATPase activity by bacterial 23S rRNA - *dbpA* (Nicol and Fuller-Pace 1995), and cold-shock adaptation - *csdA* (Jones, Mitta et al. 1996). A function has not been ascribed to a fifth *E. coli* DEAD box RNA helicase, *rhlE*; however, a null mutant grows normally under a variety of conditions, implying that *rhlE* is not an essential gene (Ohmori 1994).

RNA helicase gene (*csdA*) from *E. coli* is located at 72 min in the revised *E. coli* map (Berlyn 1998), together with other cold-shock genes. An upstream region contains the cold-shock genes *nusA* (encoding transcription elongation-termination factor), *infB* (translation initiation factor IF2) and *rbfA* (ribosome binding factor) required for translation in the cold (Jones and Inouye 1996) followed by *pnp* (polynucleotide phosphorylase - PNPase) involved in mRNA turnover and stability (Zangrossi, Briani et al. 2000) and an open reading frame (ORF) with high homology to lipoprotein NlpI (Ohara, Wu et al. 1999). It was shown that PNPase is essential for growth at low temperatures of *E. coli*, *B. subtillis* and *Y. enterocolitica* (Luttinger, Hahn et al. 1996; Goverde, Huis in't Veld et al. 1998). Expression of PNPase is post-transcriptionally autoregulated at the level of both translation and stability of the RNase III processed mRNA (Jarrige, Mathy et al. 2001). According to these data it is obvious that this region is very important for cold-shock adaptation in *E. coli*, and it would be interesting to analyze how these genes are organized and

expressed in some other organism. Helicases from other bacteria should be characterized and purified in order to develop more comprehensive picture of the varieties of prokaryotic involvement of DEAD helicases in processes like a ribosomal biogenesis, initiation of translation, or mRNA degradation.

Due to the potentially important role(s) that DEAD box RNA helicase DeaD (CsdA) may play in low temperature adaptation and due to the absence of knowledge concerning low temperature gene regulation in psychrotolerant bacteria, it is important to identify and analyze such genes involved in cold-shock adaptation of psychrotolerant organisms. Investigations on the mechanisms of low temperature adaptation in the psychrotolerant specie *Y. enterocolitica* were initiated by (Neuhaus, Francis et al. 1999; Neuhaus, Rapposch et al. 2000). Here, we report the characterization of a *deaD* gene from *Y. enterocolitica* with similarity to DEAD-box RNA helicases. The structure and phylogeny of the encoded protein is examined and the potential function of the helicase in low temperature adaptation was determined by analyzes of mRNA induction and stability during a cold-shock adaptation. EGFP transcriptional gene fusions were used to determine weather the increase in mRNA level for *deaD* at low temperature involves a transcriptional and/or post-transcriptional control mechanism.

1.6 Aims of this work

The first objective of this study was to prove specificity of mRNA cutting and specific decay of *cspA1/A2* mRNA. Primer extension experiments demonstrated that mRNAs fragments generated from the *cspA1/A2* gene have the common core sequence AGUAAA at their 5‘ends, designated like CSC-box. These findings led to a hypothesis centered on specific mRNA cleavage and degradation as a very appealing mechanism for the downregulation of *cspA1/A2* mRNA, after cold-shock induction.

The second major task was to determine the enzyme(s) involved in this mRNA decay process and further characterize the specific gene products. Among five different sequenced genes, DEAD box RNA helicase encoded by *deaD* gene was used for further detail analysis.

2 MATERIALS AND METHODS

2. 1 Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Tab. 2.1 *Escherichia coli* strain DH5αMCR (Invitrogen) and XL1 blue (Stratagene) in combination with plasmid pBluescript II SK- (Stratagene) and pEGFP (Clontech) were used for cloning and subcloning of specific fragments before electroporation in *Y. enterocolitica*. Growth media used was Luria-Bertani broth medium (LB -10 g tryptone, 5 g yeast extract and 5 g NaCl per 1l, pH 7.4). The solid media contained 1,5 % agar. Antibiotics (Sigma) were used at the following concentrations: 50 µg ampicillin ml^{-1}; 30 µg kanamycin ml^{-1}, when was necessary.

2. 2 Standard DNA manipulations

2.2.1 Isolation of genomic DNA from *Y. enterocolitica* NCTC10460

For the sequencing of the *pnp-nlp1-deaD* region *rhl*B, *eno* and *rnc* genes form *Y. enterocolitica* it was necessary to isolate and purify the genomic DNA of *Y. enterocolitica* NCTC10460. This was done according to an alternative protocol for preparation of genomic DNA from bacteria (Wilson 1987). A 5 ml culture grown overnight at 30°C was harvested by centrifugation at 10000 xg_{max} for 10 min. The pellet was resuspended in 567 µl TE buffer (10 mM Tris/HCl [pH8]; 1 mM EDTA [pH8]) containing 100 µg proteinase K in 0,5 % SDS to ensure the destruction of the cell wall. After an incubation of 60 min at 37°C, 100 µl of 5M NaCl was added, gently agitated, than 80 µl of 10 % CTAB in 0,7 M NaCl was added and incubated for 10 min at 65°C. The DNA was extracted using a phenol, (2 ×) phenol/chloroform/ isoamylalcohol (50/48/2, [v/v/v]) extraction. Finally the DNA was ethanol precipitated.

Table 2.1 Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source/reference
Strains *Y. enterocolitica* NCTC10460	Biotype 3, Serotype 1 (2a, 3)	WS – 3371 W.Frederiksen, Copenhagen (1966)
Y. enterocolitica KC1	*Y. enterocolitica* mutant for *deaD* gene generated by single crossover with plasmid pKCsdA4	WSGMO – 7080 This study
CC118	*E. coli* K12 *(λpir)*, plasmid free strain *ara*Δ139 Δ(*ara leu*) 7697 Δ*lac*X74 *pho*AD20 *gal*E *gal*K *thi rps*E *rpo*B *arg*E *am rec*A1	WSGMO – 7076 Stratagene
XL1-blue	*E. coli*, plasmid free strain *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F´ *pro*AB$^+$ *lac*I^{q}ZΔM15 Tn10 (Tet^r)]	WSGMO – 7077 Stratagene
DH5α-MCR	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80 d*lac*Z ΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *sup*E44 λ-*thi*-1 *gyr*A96 *rel*A1	WSGMO – 7078 Life Technologies
MG1693	F-, thyA715, λ-, rph-1 (polar effect on pyrE)	WSGMO – 7001 (Arraiano, Yancey et al. 1988)
SK5704	F-, thyA715, λ-, rne-1 (conditionally lethal), pnp-7, rnb-500, rph-1 (polar effect on pyrE).	WSGMO – 7008 (Arraiano, Yancey et al. 1988)
SK5665	F-, thyA715, λ-, rne-1 (conditionally lethal), rph-1 (polar effect on pyrE).	WSGMO – 7003 (Arraiano, Yancey et al. 1988)
Plasmids pBluescriptIISK- (pBSK)	3,0 kb cloning vector, f1(-) origin, ColE1ori, Amp^r	WSGMO – 7081 Stratagene
pUC4K	3,9 kb, Kan^r, Amp^r	Amersham Pharmacia Biotech
pBSK(K)1	4,25 kb, derivative of pBSK cloning vector, Km^r	WSGMO – 7085 This study
pKD1-15	4,3 kb plasmid with ATGAAA specific sequence subcloned in pBSK(K)1, Km^r	WSGMO – 7086 This study
pD5	4,3 kb plasmid with ATGCCC sequence subcloned in pBSK(K)1, Km^r	WSGMO – 7087 This study
pKNG101	6,9 kb suicide vector, sucrose marker (*sac*B), R6Kori, Str^r	WSGMO – 7039 (Kaniga, Delor et al. 1991)
pMRS101	8,7 kb improved suicide vector pKNG101, , sucrose marker (*sac*B), R6Kori, Str^r, Amp^r	WSGMO – 7040 (Kaniga, Delor et al. 1991)
pKCsdA4	pKNG101 plasmid with *Sal*I/*Xba*I PCR generated fragment of *deaD* gene, Str^r	WSGMO – 7079 This study
pKΔRhlB18	pKNG101 derivative for RhlB deletion mutagenesis (*Sal*I/*Pst*I/*Bam*HI fragment of RhlB gene), Str^r	This study
pKΔRhlB(K)2	pKNG101 derivative for RhlB deletion mutagenesis with Kan^r gene inserted in *Pst*I of pKΔRhlB18, Km^r ,Str^r	This study
pEGFP	Promoter probe vector; 4,0 kb, Km^r	WSGMO – 7093 Clontech
pLacZ-1	pEGFP plasmid containing (*Sal*I/*Bam*HI) PCR generated *lacZ-egfp* transcriptional fusion; Km^r	WSGMO – 7094 This study
pPNP-1	pEGFP plasmid containing (*Sal*I/*Bam*HI) PCR generated *pnp-egfp* transcriptional fusion; Km^r	WSGMO – 7095 This study
pDeaD-1(4)	pEGFP plasmid containing (*Sal*I/*Bam*HI) PCR generated *deaD*1-*egfp* transcriptional fusion; Km^r	WSGMO – 7096 This study
pDeaD-2(15)	pEGFP plasmid containing (*Sal*I/*Bam*HI) PCR generated *deaD*2-*egfp* transcriptional fusion; Km^r	WSGMO – 7097 This study

Table 2.2 Primers used in this study

Oligo-nucleotide	DNA sequence	Function/features	Tm-t°C
DeadF1	5‘-GGATAACACTTCGCTCGC-3‘	PCR for sequencing	56,0
DeadF50	5‘-CATTACCTAAGTCTGGGG-3‘	PCR for sequencing	53,7
DeadR1900	5‘-CCTCTTTTGCTGATCTGGG-3‘	PCR for sequencing	56,7
DeadR1400	5‘-CTGAACATCAGACCGCGC-3‘	PCR for sequencing	58,2
YpDeadF1200	5‘-GCTTCCAGGAAACGTACC-3‘	PCR for sequencing	56,0
YpDeadR1000	5‘-GGACGTTGAGCGTATCAGC-3‘	PCR for sequencing	58,8
YpDeadR400	5‘-CCGCATTGAAGTGGGCCG-3‘	PCR for sequencing	60,5
YpDeadF250	5‘-CTGCATGTTCA(GT)CGGCTTG-3‘	PCR for sequencing	57,7
DeadR6300	5‘-CTCGCGATATTCTGCGCC-3‘	PCR for sequencing	58,2
UpF600	5‘-CTGGCAGGATGCAGCAACC-3‘	PCR for sequencing	61,0
UpR1250	5‘-CGAAGCGGTGTCCTTGTCC-3‘	PCR for sequencing	61,0
PnpF3480	5‘-GGGTGACGAAGATCACTTGGG-3‘	PCR for sequencing	61,8
PnpF2400	5‘-CAAGGTCGTATCCGCCTG-3‘	PCR for sequencing	58,2
PnpR4140	5‘-CTCTGCTGCTTCTGGTGC-3‘	PCR for sequencing	58,2
Pnp8F	5‘-CCGCAGTATTCGTTACTGTTG-3‘	Amplification of ***pnp*I** probe	57,9
PnpR2700	5‘-GCTCAGCATGCCAATCCC-3‘	Amplification of ***pnp*I** probe	58,2
DeadF1200	5´-GCTTCCAGGAAACGTACC-3´	Amplification of probe ***deaD*I**	56,0
DeadR1900	5´-CCTCTTTTGCTGATCTGGG-3´	Amplification of probe ***deaD*I**	56,7
DeadR1000	5´- GGACGTTGAGCGTATCAGC-3´	Amplification of probe ***deaD*III**	58,8
DeadR2300	5´-CACCGAAACGACGACGAG-3´	Amplification of probe ***deaD*III**	58,2
Dead_*Bam*HI	5‘-TATA**GGATCC**CGGCTGAACATCA GACCG-3´	Forward primer for insertion mutagenesis with pKNG101	68,0
Dead_*Xba*I	5‘-TATA**TCTAGA**GGACGCTCGCCCT GTGCC-3´	Reverse primer for insertion mutagenesis with pKNG101	69,5
Eno1F300	5‘-GTTTCGGTCAGAGAACCG-3‘	PCR for sequencing	56,0
Eno2R650	5‘-GGCTGTTATCGCTGAAGC-3‘	PCR for sequencing	56,0
Eno4F1000	5‘-GCCAAGATGGCGTTAGCACC-3‘	PCR for sequencing	58,8
Eno3R1200	5‘-CTACTGGTTCCCGTGAAGC-3‘	PCR for sequencing	58,8
RhlBF50	5‘-GCTAAACACCCAGCTTAACC-3´	PCR for sequencing	57,3
RhlB2R200	5‘-GGGCTTCAATAACTAGCGG-3´	PCR for sequencing	56,7
RhlBchF950	5‘-GGCGTAGATATTCTTATCGG-3´	PCR for sequencing	55,3
RhlBchR1000	5‘-CTCATCCAATACCACAACC-3´	PCR for sequencing	54,5
RhlBchF1400	5‘-GGTCTATTGACCGGTGATG-3´	PCR for sequencing	56,7
RhlBR1400	5‘-CATCACCGGTCAATAGACC-3´	PCR for sequencing	56,7
RhlBchR1850	5‘-GGAGCCAAGATCGATGGC-3´	PCR for sequencing	58,2
RhlBnF350	5‘-GAAGCTGTCGTCACTCAGG-3´	Amplification of **RhlB** probe	58,8
RhlBnF600	5‘-CCGCTAGTTATTGAAGCCC-3´	Amplification of **RhlB** probe	56,7
RhlB(*Sal*I)N-F1	5‘-ATAT**GTCGAC**CCATCATCTTCAAA GGG-3´	Forward1 Primer for RhlB deletion in pKNG101	63,4
RhlB(*Pst*I)N-R1	5‘-TATAT**CTGCAG**GCGCCTGACCCG CTACATC-3´	Reverse1 Primer for RhlB deletion in pKNG101	70,9
RhlB(*Pst*I)C-F2	5‘-ATATA**CTGCAG**CAGACGTTGATTG AAGAAGAG-3´	Forward2 Primer for RhlB deletion in pKNG101	65,6
RhlB(*Bam*HI)C-R2	5‘-ATAT**GGATCC**CTTAGCATCATTTAT CAGCTCGG-3‘	Reverse2 Primer for RhlB deletion in pKNG101	67,0

continued

Tab. 2.2 ***continued***

Oligo-nucleotide	DNA sequence	Function/features	Tm-t°C
RncF1	5‘-CGGTAACGTTGAATTACCGC-3´	PCR for sequencing	57,3
RncChR1500	5‘-GAGTCTTGTTATCAACCGC-3´	PCR for sequencing	54,5
RncF2	5‘-CGAACCCTTCCAGATTCC-3´	PCR for sequencing	56,0
RncR200	5‘-CCGAGGGTTTCGGTACGC-3´	PCR for sequencing	60,5
RncCh550	5‘-CCCGCAGGGTATTTAAACGC-3´	PCR for sequencing	59,4
RncChR650	5‘-CTGTTTCGTTTGCAGTGGC-3´	PCR for sequencing	56,7
RncF50(I)	5‘-GGCTGCAACGGAAGCTGGG-3´	PCR for sequencing	63,1
RncF1100	5‘-GAACGGCTGATTCTCGCC-3´	PCR for sequencing	58,2
RncChR1250	5‘-GGTAATGGCAGATGACGG-3´	PCR for sequencing	56,0
RncR2200	5‘-GCTTCCGGCATATGCTTGC-3´	PCR for sequencing	58,8
YeA2R3(+1)	5‘-CCTAATCACACTGAATTACAGG-3‘	primer extension – *cspA1/A2*	NA
YeA1R2(+1)	5‘-GCCACAATACTGTTTTGCCAC-3‘	primer extension – *cspA1/A2*	NA
Pnp21R	5‘-TGTTGGCCGTACTGGAATTTA-3‘	primer extension – *pnp*	NA
Pnp7R	5‘-GACGTGAAGTCAGTGTTTCG-3‘	primer extension – *pnp*	NA
DeadR31	5‘-GATACAGCCACAACGAACG-3‘	primer extension – *deaD*	NA
DeadR32	5‘-GTGCCCAGGTAGAATTCG-3‘	primer extension – *deaD*	NA
DeadR400	5‘-CCCAGATCAGCAAAAGAGG-3‘	primer extension – *deaD*	NA
DeadR37	5‘-CAGTCAGAGCAGAAAGAATTGG-3‘	primer extension – *deaD*	NA
RhlBR50	CCGGTGAAATAAAGTCATCC	primer extension – *rhlB*	NA
RhlBR600	GATCGGCGTGCAATACTG	primer extension – *rhlB*	NA
RhlBR750	GTTGATTAGTCTGACGACC	primer extension – *rhlB*	NA
Rnc1350	CCCAGCTTCCGTTGCAGCC	primer extension – RNase III	NA
PlacZ_F(*Sal*I)	5‘-TATA**GTCGAC**CGACCGAGCGCA GCGAGTCAG-3‘	Amplification of *lacZ* promoter	72,3
PlacZ_R(*Bam*HI)	5‘-AGCT**GGATCC**TGTGTGAAATTGT TATCC-3‘	Amplification of *lacZ* promoter	63,7
Ppnp_F(*Sal*I)	5‘-TATG**GTCGAC**TACCTGAAGCGTA AAGATG-3‘	Amplification of *pnp* promoter	65,3
Ppnp_R(*Bam*HI)	5‘-TATA**GGATCC**TTATACTATGCGG TGCCAG-3‘	Amplification of *pnp* promoter	65,3
Pcsd_F1(*Sal*I)	5‘-ATAT**GTCGAC**GTGACAGCACTCA TGCTG-3‘	Amplification of *dead1* promoter	66,6
Pcsd_R1(*Bam*HI)	5‘-TAAC**GGATCC**GAAAAATCATTTC GCGC-3‘	Amplification of *dead1* promoter	63,4
Pcsd_F2(*Sal*I)	5‘-TATA**GTCGAC**GGATAACACTTCG CTCGC-3‘	Amplification of *dead2* promoter	66,6
Pcsd_R2(*Bam*HI)	5‘-TATA**GGATCC**AGTCTACATAACT CGTCG-3‘	Amplification of *dead2* promoter	63,7
EGFP_F(2)	5‘-GTTATTACTAGCGCTACCG-3‘	pEGFP specific 5’-Primer	54,5
EGFP_R(170)	5‘-GTGGCCGTTTACGTCGCC-3‘	pEGFP specific 3’-Primer	60,5

*Restriction sites (*Sal*I and *Bam*HI) introduced at the 5’ and of the oligonucleotides are in bold type. NA, not applicable (primers used for primer extension analysis with 5’-IRD800 labeling).

2.2.2 Isolation of plasmid DNA

Plasmid DNA was isolated from plasmid-carrying *E. coli* or *Y. enterocolitica* cultures, after overnight incubation in 20 ml liquid culture. Three to five ml aliquots were taken for isolation of plasmid DNA and further proceeded according to instructions for GeneEluteTM Plasmid Mini-Prep Kit (Sgma).

2.2.3 Digesting DNA with restriction enzymes

Restriction digestion was performed by standard methods (Sambrook J 1989) and according to instructions from manufacturers for restriction enzymes (Promega, Biolabs, MBI etc.).

2.2.4 DNA precipitation by Ethanol / Isopropanol

DNA in a sample was precipitated by the addition of 0.3 M sodium acetate (pH 5.2, 0.1 volume of 3 M) along with 2 to 2.5 volumes of ethanol (-20°C) and incubation for 30 min at -20°C. The sample was then centrifuged at 4°C maximum speed) in a microcentrifuge (Eppendorf) and washed once with 70 % ethanol. After a second centrifugation the pellet was left to dry and finally resuspended in MilliQ water or TE buffer. Instead of using 2/2.5 volumes of ethanol 1 volume of isopropanol can also be used for precipitation of DNA.

2.2.5 Separation of DNA fragments by agarose gel electrophoresis (AGE)

To prepare a 1 % gel, 0.5 g agarose (SeaKem LE, FMC BioProducts) were dissolved in 50 ml 1 x TAE buffer using a microwave. The hot agarose solution was poured into a gel tray with slot-formers. 5 µl of DNA containing samples were mixed with 1 µl 6x gel loading buffer (GLB) Blue/orange 6x loading dye (Promega) and pipetted into the gel slots. Additionally an appropriate molecular weight marker was loaded. After electrophoresis, the gel was stained with ethidiumbromide EtBr (0.5 µg ml^{-1}) for 10-15 min and visualized by UV-transillumination using the ImageMaster® VDS (Pharmacia, Biotech). Electrophoresis was performed using the following equipment: Easy-Cast™ Electrophoresis system (Owl Scientific Inc. PeqLab) and Electrophoresis Power Supply EPS 600 (Pharmacia Biotech).

TAE (Tris/acetate/EDTA) electrophoresis buffer- 50 x stock solution (1 l):

2 M Tris-base
1 M NaAc
50 mM EDTA (pH 8.0)

Gel loading buffer: (GLB 6x):

0.10 % Xylen-Cyanol FF (XCFF)
0.25 % Bromophenolblue
0.20 % SDS
5 mM EDTA
50 % (v/v) Glycerol
(pH 8.0)

2.2.6 Preparation of DNA fragments from agarose gels

Gel extraction was performed by using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer.

2.2.7 Ligation

Ligations were performed using 1 U T4 DNA ligase (Promega) and 10x reaction buffer (Promega) for 4 h at 21°C or for 18 h at 4°C.

2. 3 Construction of plasmids for *cspA1/A2* mRNA processing analysis

E. coli XL1blue strain was used for subcloning experiments. Because of the natural resistance of *Y. enterocolitica* to ampicillin, a pBluescript II SK$^-$ (Stratagene) was digested with *ScaI* and a kanamycin resistance (kan^R) cassette was introduced yielded and pBSK(K)1 plasimd. The kan^R cassette (*Eco*RI*/ Eco*RI fragment blunt-end filled with Klenow fragment (Boeringer)) was used from pUC4K plasmid (Amersham Pharmacia Biotech). Synthetic DNA fragments (sequence data derived from *cspA1/A2* of *Y. enterocolitica*) were ligated into the *EcoRV* site of this modified vector (pSK(K)1) and two different plasmids (pKD1 and pD5) were obtained. The artificial sequence for pKD1 was 5´-GAT GTC TAA TAA AAT GAC TGG TTT **AGT AAA** ATG GTT TGA CGC TGG TAA AGG TTT ATC-3´, with the CSC-box presented in bold letters. It was impossible to obtain correct construct during subcloning experiments in *E. coli* cells, since all plasmids sequenced for insertion were mutated. We have chosen pKD1-15, a deletion mutant that has no influence on the CSC-box sequence since the mutation occurred downstream of the CSC-box motif. The missing base is marked with an asterisk: 5´-GAT GTC TAA TAA AAT GAC TGG TTT **AGT AAA** ATG GTT TGA CGC TGG TAA *GG TTT ATC-3´. As a control, a fragment was created which has an altered CSC-box sequence, but exhibits the same global folding. The folding studies of the mRNA were conducted

with *mfold* version 2.3 (Zuker 1989; Mathews, Sabina et al. 1999). For pD5, the artificial sequence 5´- GAT GTC TAA TAA AAT GAC TGG TTT **AGT <u>CCC</u>** ATG GTT TGA CGC TGG TAA AGG TTT ATC-3´ was chosen (the altered CSC-box is presented in bold letters and the changes are underlined). The plasmids obtained were purified by standard methods (Sambrook J 1989) and sequenced using a SequiTherm EXCEL^{TM} II DNA-LC sequencing kit (Epicentre Technologies) with a fluorescent-labelled standard primer (M13) and reaction products were analyzed on a Li-COR sequencer (MWG-Biotech).

Y. enterocolitica was transformed with pKD1-15 and pD5 by electroporation. The transformants were grown in presence of kanamycin (50 μg ml^{-1}) and IPTG (1mM) in order to induce synthesis of the *lacZ*' mRNA containing the *cspA1/A2* insertion fragments. Total RNA was isolated after cold-shock experiments and analyzed with primer extension reactions for mRNA processing products. Band intensities were measured and calculated by using ImageMaster 1D Elite Version 3.00 (Amersham Pharmacia Biotech).

2. 4 Electrotransformation

Transformation of *E. coli* and *Y. enterocolitica* was carried out by electroporation according to the protocols by (Dower, Miller et al. 1988) using a BioRad Gene Pulser and sterile, individually wrapped 2-mm electroporation cuvettes (EquiBio Ltd., Kent, UK). Selection for resistance to antibiotics in *E. coli* and *Y. enterocolitica* was performed with ampicillin (100 μg ml^{-1}) and kanamycin (50 μg ml^{-1}). Colonies harboring recombinant plasmids were selected according to standard methods (Sambrook J 1989).

2.3.1 Preparation of competent cells

300 ml of LB broth were inoculated with 5 ml of an overnight liquid culture of the *E. coli* or *Y. enterocolitica* and incubated at 37°C shaking at 150 rpm. After reaching an optical density of OD600 0.5-0.6 cells were harvested by centrifugation (8000 × g, 4°C, 10 min) and stored on ice. Cell Pellet was resuspended and washed with: 300 ml 5 % glycerol (4°C), 150 ml 5 % glycerol (4°C), 10 ml 10 % glycerol (4°C). Finally the pellet was resuspended in 1 ml 10 % glycerol (4°C) and stored in 50 μl aliquots at -70°C.

2.3.2 Electroporation

20 µl of the ligation mixture was dialyzed for 30 min using dialysis membrane filter (0.025 µm pore size, Millipore) or 1 µl of plasmid were electoporated into 50 µl of *E. coli* or *Y. enterocolitica* competent cells (see 2.3.1) on ice using a Gene-Pulser (BioRad) under the following conditions: Resistance: 200 □□Capacitance: 25 µFD; Volt: 2.5 kV (12.5 kV/cm); Time constant: ~4.7. Following electroporation, 500 µl LB-medium was added directly to the electroporation cuvettes. For regeneration of the host cells, the mixture was incubated at 37°C for 1 h. 100 µl aliquots (and ten-fold dilutions) were plated on LB plates supplemented with the corresponding antibiotic. Plates were incubated for 18 h at appropriate temperature.

2. 5 Polymerase Chain Reaction (PCR)

Oligonucleotides used for PCR reactions were obtained from MWG Biotech (Ebersberg, Germany). PCR amplification was performed with a Techne Progene or MWG Biotech automated thermocycler with 0.2-ml thin-walled PCR tubes (Advanced Biotechnologies). Reactions were carried out in 50 µl volumes containing 5 µl 10x PCR reaction buffer (containing 2.0 mM $MgCl_2$), 100 pmol of each oligonucleotide primer, 2mM dNTP mix containing eqimolar concentration of all four nucleotides (dNTP, Eurogentech) and 0,5U of *Taq* DNA polymerase (Eurogentech or ABgene). The following PCR conditions were used: initial denaturation step at 95°C for 5 min; amplification was carried out with 25-30 cycles (denaturation at 95°C for 15s, annealing at 50°C for 20s and extension at 72°C for 30s) followed by a final extension step at 72°C for 5 min. The annealing temperature was adjusted to the melting temperature of the primers and the extension time to the size of the expected fragment.

2.4.1 Inverse PCR

Inverse PCR was performed as described by Ochman *et al*., (Ochman 1990) for the in vitro amplification of the upstream and downstream flanking regions of the known sequence. According to the sequence alignment of *csdA* gene from *E. coli* with sequence database of *Y. pestis* and *Y. enterocolitica* from the Sanger Institute, set of primers were designed for sequencing a *pnp-nlp1-deaD* locus in *Y. enterocolitica* (primers are listed in Tab. 2.2). Chromosomal DNA of *Y. enterocolitica* was digested at 37°C for 3 h with the restriction endonucleases

*Bam*HI, *Eco*RI, *Sal*I, *Kpn*I, *Not*I, self-ligated with T4 DNA ligase (Promega) and used as a template for PCR amplification with specific primers derived from the high sequence homology between *E. coli* and *Y. enterocolitica* in analyzed region.

2.4.2 Purification of PCR products

PCR products were purified using spin columns with the QIAquick-spin PCR Purification Kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer.

2. 6 DNA sequencing

Double-stranded DNA fragments from PCR reactions and different plasmids were sequenced by the dideoxy-chain-termination method using fluorescent-labeled sequencing primers and a SequiTherm EXCEL^{TM} II DNA-LC sequencing kit for 66-cm gels (Epicentre Technologies). Primers were 5´-end-labelled with IRD-800 (MWG Biotech) and sequencing products were analyzed on a Li-COR automated DNA sequencer (MWG-Biotech). DNA sequence determination was also performed by using a Dydeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and reaction products were analyzed using an Applied Biosystems model 373A automatic DNA sequencer according to the manufacturer's protocol (W.Metzger Sequiserve). The sequences of individual fragments were assembled to obtain the nucleotide sequence of a 6253 bp fragment that contained the entire *pnp-nlp1-deaD* locus from *Y. enterocolitica*. Specific sequencing reactions were used during Primer extension experiments like a ladder.

2. 7 Cold-shock experiments

350 ml batch cultures of LB were inoculated with *Y. enterocolitica* and grown at 30°C, to an optical density of 0.5 at 600nm on a shaker (150 rpm). The cultures were then cold shocked to 10°C in an ice-bath. Samples (10ml) were taken immediately before (control, 0 min) and after (2 min) cold shock, as well as after different times subsequently to cold shock. The cells were centrifuged (10.000*g*; 2 min at +4°C) and the pellet was frozen in liquid N_2.

2. 8 Isolation of total RNA

Total RNA was isolated in a guanidine-phenol buffer as described (Grsic, Sauerteig et al. 1998). Cell pellets from cold-shock experiments were dissolved in 640 µl of Extraction buffer, than mixed with glass beads (Roth) in Ribolyser (Hybaid) for 30 seconds on maximum speed. Following 320 µl of chloroform was added and mixed for 10 min on ice. The sample was then centrifuged at 4°C (at maximum speed 13,000 rpm) in a microcentrifuge (Eppendorf) and the supernatant was transferred to a fresh tube. The RNA was extracted using a phenol, (2X) phenol/chloroform/isoamylalcohol (50/48/2, [v/v/v]), (13,000 rpm, 4°C, 10 min). Upper phase was mixed with an equal volume of Complex Buffer/Chloroform (1vol/1vol) and mixed for 20 min on ice and than centrifuged (13,000 rpm, 4°C, 20 min). Finally RNA was ethanol precipitated.

Extraction buffer:

Guanidin-HCl	8M	76,42g/100ml
MES	20mM	0,39g/100ml
EDTA	20mM	0,74g/100ml
(pH 7.0 with 25 % HCl)		

Complex buffer:

CTAB	3%	3,0g/100ml
1M NaCl	1M	5,8g/100ml
0,2M Tris-HCl	0,2M	3,2g/100ml
0,05M EDTA	0,5M	1,9g/100ml
(pH 7.0 with 5M NaOH)		

Solutions for RNA isolation were prepared using DEPC water:
0,1 % DEPC (Diethylpyrocarbonate, Roth) (0,1ml/100ml MilliQ). After shaking the solution and incubation for 12 h at 37°C, the solution was autoclaved for 15 min to remove traces of DEPC.

2.7.1 Photometric purity control and quantitative determination

The concentration of the RNA was determined spectrophotometrically by measuring the absorbance at 260nm (A260) and 280nm (A280). 1 µl of RNA sample was diluted in 500 µl MilliQ and measured against water. The quality of each RNA preparation was checked by visualization of rRNA bands in ethidium-bromide stained agarose gels that were electophoresed under denaturing conditions.

2. 9 Northern hybridization and blotting

For transcriptional RNA analysis, total RNA (15 µg samples) was denatured at 65°C for 15 min in the presence of 2M formaldehyde and 50 % formamide. RNA was then separated on a formaldehyde (MOPS) agarose gel and transferred to a nylon-membrane (Hybond N+, Amersham Pharmacia). Northern-blots were carried out according to Löw and Rausch (1994) with minor changes as described (Neuhaus *et al*., 1999). Formaldehyde (MOPS) 0,8 – 1 % agarose gels were made in 40 ml MilliQ/DEPC water with 2 ml (20XMOPS) and 6,6 ml Formaldehyde.

20x MOPS

MOPS	0,4 M	8,37g/100ml
NaAc	0,1 M	1,36 g/100ml
EDTA	20 mM	0,74 g/100ml
(pH 7.0 with 5M NaOH)		

2.8.1 Preparation of specific DIG-labelled PCR probes

Digoxigenin labelled DNA probes were obtained by PCR amplification of chromosomal DNA from *Y. enterocolitica* using digoxigenin labelled DIG-11dUTP nucleotide (Roche Diagnostics GmbH). Primers used for the PCR probe I which covers a coding region at the 5' end of the *csdA* mRNA, were YeF1200 and YeR1900. For the probe III which covers coding region at the 3' end of the *csdA* mRNA, primers YeR1000 and YeR2300 were used, and Ye8F and YeR2700 primers generated *pnp*l probe. All primers are listed in Tab. 2.2. PCR-generated DIG labeled DNA fragments were recovered from agarose gels. The gel slice was dissolved in 20 ml prehybridization buffer using a microwave**.**

Preparation of DIG-dNTP Mix:

1µl dATP (2mM)
1µl dCTP (2mM)
1µl dGTP (2mM)
0,6µl dTTP (2mM)
46,4µl MilliQ
ad 50µl

25µl DIG-dUTP (1,3mM)
ad 75µl

PCR reaction mix for DIG labelled probe:

5µl 10x PCR reaction buffer
6µl DIG labelled dNTP mix
4µl MgCl2 (2mM)
1µl $Primer_{forward}$ (2pmol)
1µl $Primer_{reverse}$ (2pmol)
0,1µl DNA template
1µl Taq polymerase (1U)
ad 50µl

2.8.2 Northern hybridization

Hybridization was carried out overnight at 50°C with specific DIG-labelled probes by using a Micro-4 hybridization oven (Hybaid). Hibridized probes were detected by chemoluminescence using an AGFA Curix X-Ray film (AGFA-Gevaert) which was exposed for different time intervals (10-60 min).

After hybridization, band intensities were measured and calculated by using ImageMaster 1D Elite Version 3.00 (Amersham Pharmacia Biotech).

20x SSPE

NaCl	3M	174g/L
$NaH_2PO_4 \cdot H_2O$	220 mM	27.6 g/L
EDTA	20 mM	7.4 g/L
(pH 7.4 with 5M NaOH)		

10x PBS

$Na_2PO_4 \cdot 2\ H_2O$	580 mM	71.2 g/L
$NaH_2PO_4 \cdot H_2O$	170 mM	13.9 g/L
NaCl	680 mM	58.4 g/L

(Pre)hybridization solution:

Formamide	50%	10 ml
SDS	1%	1 ml 20% SDS
NaCl	1 M	4 ml 5M NaCl
PEG 6000	6%	1.2 g
$H_2O_{bidest.}$	-	4 ml
Lachssperma-DNA	250 µg/µl	1 ml stock (5 mg/ml)

Table 2.3 Reaction steps, solutions and buffers for Northern hybridization

Reaction step	Solutions / Buffers	time	temperature
I. Hybridization			
	1. Prehybridization buffer	30 min–2 h	50°C
	2. Dig-labelled probe	over night	50°C
II. Washing			
1. Low stringent 2x SSPE 0.5% SDS	50 ml 20x SSPE 12.5 ml 20% SDS ad 500 ml	2 x 10 min	50°C
2. High stringent 0.2x SSPE 0.5% SDS	5 ml 20x SSPE 12.5 ml 20% SDS ad 500 ml	25 min	50°C
3. Blocking reagent (Boehringer Mannheim) 1x PBS 0.5% SDS 0.2% I-Block (Casein)	50 ml PBS 12.5 ml 20% SDS 1g Block powder ad 500 ml	20 min	30°C
III. Immuno reaction			
conjugate buffer	10 ml Blocking buffer 2 µl Anti-Digoxigenin-AP Fab fragment (Roche)	1 h	30°C
IV. Washing buffer 1 x PBS 0.5% SDS	50 ml 10x PBS 12.5 ml 20% SDS ad 500 ml	2 x 5 min	30°C
V. Detection			
Reaction buffer (0.1 M DEA pH 9.5)	5g DEA (Diethanolamine) 0.5ml 1M $MgCl_2$ ad 500 ml	2 x 5 min	30°C
Substrate buffer	1 ml reaction buffer DEA 10 µl Tropix CDP-Star (Stratagene)	3 min	RT

2. 10 Primer extension analysis

Primer extension (PE) reactions were performed as described by (Loessner and Scherer 1995) with minor differences. RNA preparations were subjected to an additional treatment with RNase free DNase I (Promega). Primers used for primer extension reactions were IRD-800 labelled and they are listed in Tab. 2.2. Total RNA (5 µg) was incubated with 1 µl of 10 pmol primer for 5 min at 75°C and then

for 10 min at 50°C. Primer extension reactions were carried out with SuperScript II Reverse Transcriptase (Invitrogen) at 42°C and after 60 min the reaction was stopped by 5 min incubation at 95°C. The reaction products were then separated through polyacrylamide sequencing gels containing 6M urea and electrophoresis was carried out with a Li-COR system (MWG-Biotech, Ebersberg, Germany). For all primer extensions, sequencing reactions for ladders were carried out from different plasmids or PCR products with appropriate IRD-800 labelled primers (listed in Tab. 2.2), according to the manual for the Li-COR system.

2. 11 *In vitro* RNase E and RNase G cleavage of RNA oligonucleotides and total mRNA

The catalytic RNase E domain was overexpressed with a his-tag in *E. coli* and purified by immobilized metal affinity chromatography (IMAC) as described (Kaberdin, Walsh et al. 2000). RNA oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany) with the sequences 16mer-2cut (5´-UUU **AGU AAA** AUG GUU U-3´), and 16mer-3cut (5´-CCA **AGU AAA** UUA AGC A-3´) derived from the *cspA1/A2* sequence of *Y. enterocolitica*. Labelling of the 5´-end was conducted using T4 polynucleotide kinase and an excess of γ-^{32}P-ATP as described (Kaberdin, Walsh et al. 2000). Cleavage reactions of 0.1 nM oligonucleotide were performed with 7 nM catalytic RNase E domain as described (McDowall and Cohen 1996; Kaberdin, Walsh et al. 2000). The 1 nt ladders in Fig. 3.5 were prepared by partial digestion of 5´ labelled RNA oligonucleotides 16mer-2cut and 16mer-3cut with S1 nuclease in buffer provided by the vendor (MBI Fermentas).

5X Cleavage buffer (1ml):

Tris-HCl	1M (pH 8.0)	100µl
MgCl2	1M	25µl
NaCl	1M	500µl
Glycerol	100%	250µl
Triton X-100	100%	5µl
DTT	0,1M	5µl
MilliQ/DEPC		115µl

2.11.1 Cleavage conditions for RNase E and RNase G in reaction with total mRNA isolated after cold-shock:

Reactions were prepared in 50 µl with 10 µg total mRNA (DNaseI treated) isolated 30 and 60 min after cold-shock with 2 µl of RNase E; or Rne402 (RNase E catalytic domain) or CafA (also known as RNase G). 1 – 2 µl of RNase is usually sufficient to cleave 1 – 2 pmol of 9S RNA within 30 minutes at 30°C.

2. 12 Construction of transcriptional *egfp* fusions

The *egfp* fusions were obtained by ligating PCR fragments of different lengths, derived from the *pnp-nlp1-deaD* locus from genomic DNA of *Y. enterocolitica*, to the promoterless *egfp* gene in pEGFP-1 plasmid (Clontech). (Detailed part of genomic DNA used for appropriate fusion is presented on Fig. 3.12). All forward primers for specific PCR reactions contained a *Sal*I restriction site, and reverse primers contained *Bam*HI site for subcloning a PCR fragments in pEGFP plasmid digested with *SalI/Bam*HI enzymes. Used primers are listed in Tab. 2.2. Fusion *pnp-egfp* was obtained by subcloning a 324bp PCR fragment, which contained the *pnp* promoter. Than *deaD1-egfp* and *deaD2-egfp* fusions were constructed by subcloning a 233bp and 413bp PCR fragments from appropriate regions marked on Fig. 3.12, respectively. Control *lacZ-egfp* fusion was constructed by subcloning an 251bp PCR fragment obtained from pBlueScript SK(-) vector which contains an *lacZ* promoter. Primers used for generating an appropriate PCR fragments are listed in Tab. 2.2.

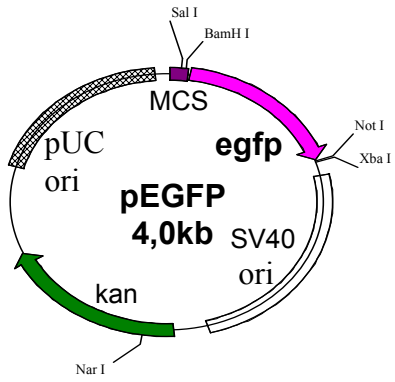


Fig. 2.1 pEGFP plasmid map used for construction of transcriptional *egfp* fusions

2. 13 *In vivo* fluorescence measurements

Y. enterocolitica cells with different plasmids for EGFP expression analysis were incubated at 30°C and cold shocked to 10°C and samples were taken at certain time points indicated in Tab. 3.1. Cell pellets from 5 ml aliquots were collected as described for cold-shock experiments above and resuspended in 0,5 ml of PBS (phosphate buffered saline). This step reduced the background fluorescence about ten-fold compared to directly measured EGFP fluorescence of *Y. enterocolitica* in LB medium. The cell suspensions (0,25 ml) were than analyzed by two parallel fluorescence measurements with the Wallac Victor 2 plate counter (Wallac, Turku FN). EGFP was excited at 485nm, and emission was detected using a 520 bandpass filter. Parallel to fluorescent measurements, cell densities were determined photometrically at 405nm with Wallac Victor2 plate counter (Wallac, Turku FN). The ratio of fluorescence intensity to cell density was taken as a measure for EGFP expression levels and represented in Relative Fluorescence Units (RFU).

Phosphate buffered saline (PBS)

Na2HPO4	58mM	8.2 g/L
NaH_2PO_4	17mM	2.4 g/L
NaCl	68mM	4.0 g/L
(pH 7.4 with 5M NaOH)		

2. 14 Construction of specific plasmids for insertion mutagenesis in *deaD* gene and *rhlB* gene

Constructs for mutagenesis by homologous recombination of a two specific DEAD box RNA helicases (*deaD* and *rhlB*) were made by using *E. coli* CC118 strain and suicide vector pKNG101 described earlier (Kaniga, Delor et al. 1991).

To construct a suicide plasmid carrying a specific part of *deaD* gene from *Y. enterocolitica,* fragment was generated by PCR with oligonucleotides Dead_*Bam*HI and Dead_*Xba*I (see Tab. 2.3) introducing *Bam*HI and *Xba*I restriction sites at the ends and yielding a 387 bp PCR fragment. After digestion with specific restriction enzymes fragment was inserted in pKNG101 (*Bam*HI/*Xba*I digested) plasmid and construct pKCsdA4 was obtained with selection on streptomycin (see Appendix for plasmid map).

Suicide plasmid carrying specific part of *rhlB* gene was made by PCR using oligonucleotides listed in Tab. 2.3. Two PCR fragments were obtained *Sal*I/*Pst*I

(653 bp) and *Pst*I/*Bam*HI (565 bp) with proper restriction sites generated at the ends. The PCR fragments were digested with *Pst*I, ligated and ligation mixture was used to generate one big PCR fragment (1218 bp) for subcloning in *Sal*I/*Bam*HI digested pKNG101 plasmid. By selection on streptomycin construct pKΔRhlB18 was isolated. Restriction map of the construct is presented in Appendix. The kanamycin resistance cassette (*Pst*I/*Pst*I fragment) was used from pUC4K plasmid (Amersham Pharmacia Biotech) for subcloning in pKΔRhlB18 over *Pst*I PCR generated restriction site. By selection on kanamycin plasmid pKΔRhlB(K)2 was isolated.

Correct plasmids were checked with PCR amplification and nucleotide sequencing and further used for insertion inactivation (gene disruption) in wild type *Y. enterocolitica* strain.

Derivatives of pKNG101 plasmid could replicate in bacterial hosts supplying *in trans* the π-protein encoded by *pir* gene, such as *E. coli* strains lysogenized with a *pir* transducing λ phage. Since pKNG101 derivatives cannot replicate in *Y. enterocolitica*, a single homologous recombination event between the *deaD* or *rhlB* part of the gene present on specific plasmid and the functional gene present on the chromosome will integrate the entire plasmid into the genome governing a streptomycin resistance for *Y. enterocolitica* mutant cells. Plasmid pKNG101 also contains *sacB* gene that encodes levansucrase from *Bacillus subtilis*. Synthesis of this enzyme is induced by sucrose. Production of levansucrase in several Gram$^-$ genera is lethal in the presence of 5 % sucrose and according to that *sacB* gene could be used as a positive marker for the excision of the vector and second homologous recombination event.

Competent cells of the wild type *Y. enterocolitica* were transformed by using the suicide plasmids (pKCsdA4, pKΔRhlB18 and pKΔRhlB(K)2) and transformants were selected on streptomycin (50 μg ml^{-1}). Homologous recombination was verified by PCR and was only successful with plasmid pKCsdA4 yielding to *Y. enterocolitica* KC1 mutant strain. Other plasmids for RhlB insertion mutagenesis should be used in future experiments.

2. 15 Bioinformatics

The program DNASIS and the Husar Analysis Package (version 4.0; http://genome.dkfz-heidelberg.de) were used for analysis of the nucleotide and amino acid sequences. The Blast algorithms (Altschul, Madden et al. 1997) were used for similarity searches in the database available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Computer modeling with the *mfold* program of M. Zuker (http://www.bioinfo.rpi.edu/applications/mfold) (Mathews, Sabina et al. 1999) was used to predict probabilities to form a specific mRNA structures in *pnp-nlp1-deaD* locus.

2. 16 Nucleotide sequence accession numbers

The DNA sequence reported here will appear in the EMBL, GenBank and DDBJ databases under accession no. AF542976 for *pnp-nlp1-deaD* locus; AF542975 for *rnc* gene. Sequences for *rhlB* gene and for *eno* gene are enclosed in appendix.

3 RESULTS

3. 1 Identification of specific recognition sites for cleavage of CSP mRNA

Primer extension experiments with primers labelled on Fig. 3.1A demonstrated that 5 different mRNA fragments originating from the *cspA1/A2* gene (except the transcription start +1) have 5' end located in the same motif. This motif has the same core sequence, AGUAAA, with the underlined A being the 5´ end of most of the resulting mRNA cleaved fragments. Primer extension with primer YeA2R3(+1) detects +1 transcriptional start site for *cspA1/A2* in *Y. enterocolitica* and the fragments named “cutting site 1” and “cutting site 2”. These were chosen as examples to show the increase in signal intensity of 5´ends at different time points after cold-shock (Fig. 3.1B). The signal observed immediatelly after cold-shock was weak compared to the signal after 1 h of incubation at 10°C. Similarly, Northern blot analysis with the A2-probe (Neuhaus, Rapposch et al. 2000) showed a decrease in the amount of the full-length transcript and an accumulation of degradation products, both occurring in line with the duration of the cold shock. It was proposed that the differently sized fragments are due to a specific cutting within the AGUAAA core sequence during *cspA1/A2* mRNA decay, which occurs at the time of restart of growth at low temperature, approximately 80 min after cold-shock.

3. 2 The AGUAAA consensus sequence is a specific cut site

We wanted to verify whether the consensus sequence found in the above primer extension experiments is indeed a specific cut site. For this purpose, a 50 bp fragment containing the “cutting site 2” of *cspA1* was introduced in frame into the α-fragment of the *lacZ* gene. For unknown reason it was not possible to obtain a construct that was completely in order with proposed sequence of 50bp. Therefore, we used a mutant with a point mutation, causing a frame shift downstream of the AGUAAA motif (see Material and Methods). The transcription of the chimerical RNA was constantly induced with IPTG. Primer extension analysis of total cellular RNA extracted at different time points after a cold-shock at 10°C (Fig. 3.2A) identified a clear 5´end signal within the AGUAAA motif even in the control sample at 30°C before cold-shock.

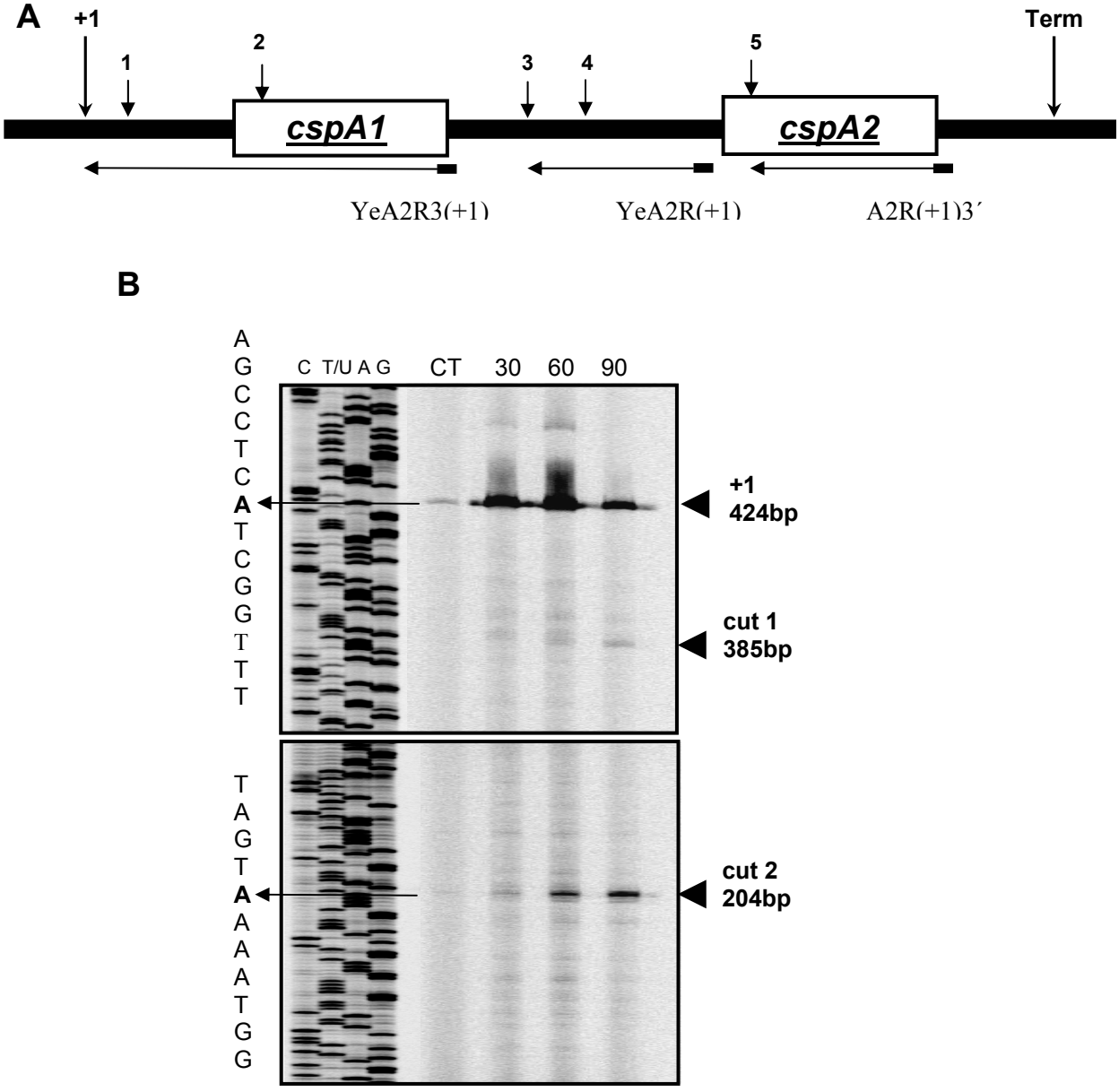
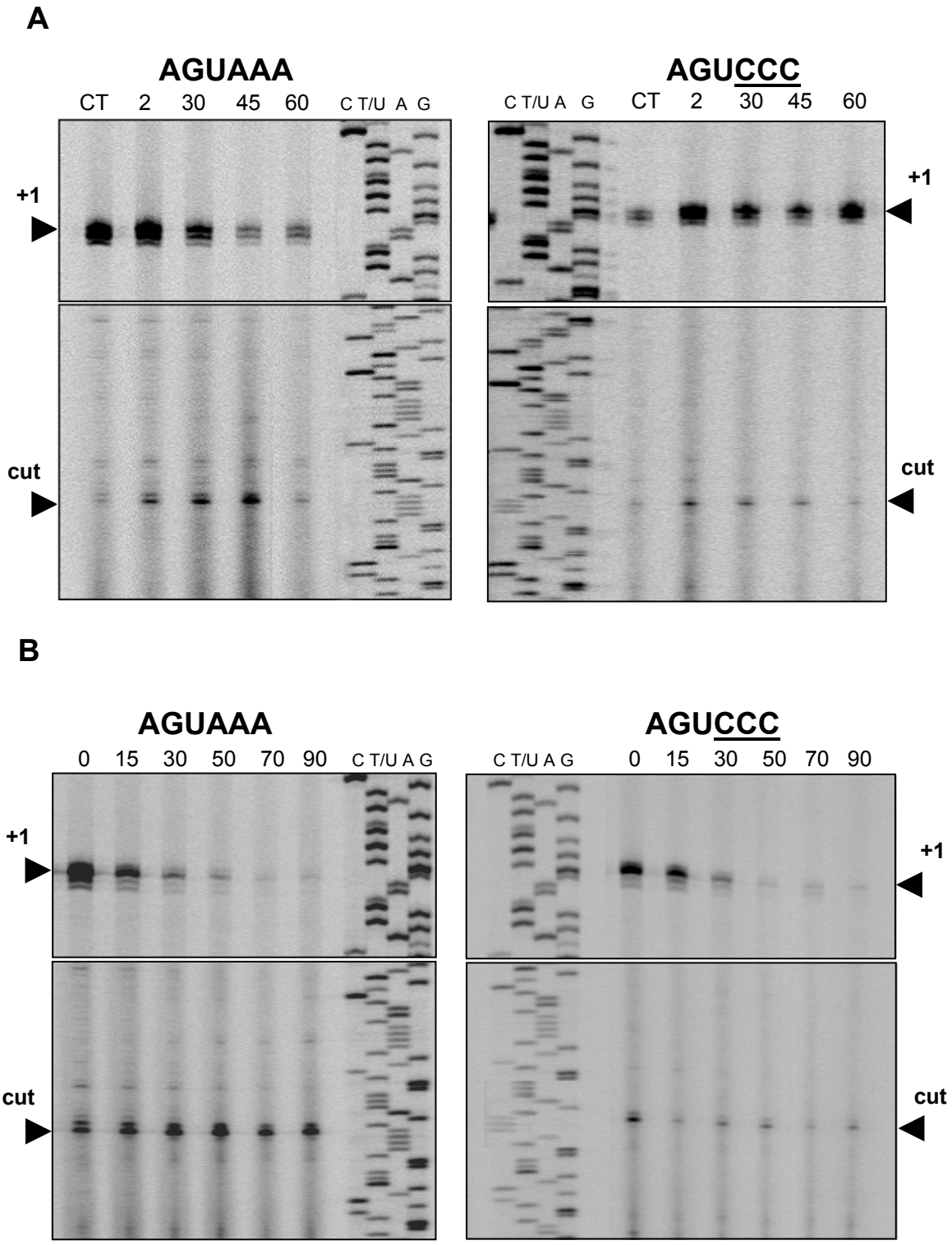


Fig. 3.1 Primer extension experiment mapping 5´ends of *cspA1/A2* fragments. Panel **A**: Organization of *cspA1/A2* gene tandem in *Y. enterocolitica* and primers used for primer extension analysis also in previous studies (Neuhaus 2000). The vertical arrows indicate the 5´ends detected (+1 transcriptional start site, 5 different cutting sites and the putative termination site). The horizontal arrows show the reverse transcription from the primers indicated (bold line at the end of the arrow). Panel **B**: Primer extension analysis of total mRNA from *Y. enterocolitica* cold shocked for 0 (CT), 30, 60 and 90 min at 10°C was done with YeA2R3(+1) IRD800 labelled primer. Lanes marked with C, T/U, A and G show sequencing reactions of the *Y. enterocolitica* DNA with the same primer. Sequencing reactions were used for precise determination of detected bands in basepares (bp) labelled. The location of the +1 site is the same detected previously with another primer extension method using ^{35}S (Neuhaus, Francis et al. 1999). The cut sites designated “1” and “2” from part 3.1A are indicated also on part 3.1B like detected bands in bp. An increase of the band intensity shows that the *cspA1/A2* mRNA undergoes degradation.

The cutting site occurred exactly at the same position in the AGUAAA sequence (the underlined A is the 5´end observed) as was found for this particular sequence in its natural setting, i.e. in the *cspA1/A2* mRNA. Cleavage of this artificial mRNA increased as rapidly as 2 min after cold shock. This signal increased after cold-shock and was strongest after 45 min. The band originating from the cutting site decreased to the pre-shock level after 60 min. Note, that +1 signal on the left part of the panel is decreasing, which is due to strong degradation as well as a low rate of synthesis of the *lacZ* mRNA due to cold shock.

The AGUAAA motif was changed to AGUCCC, and the same cold-shock experiments as discussed above were conducted on this modified mRNA. This change was chosen after examination of the mRNA folding pattern using the software program 'mfold' version 2.3 (Mathews, Sabina et al. 1999) in order to minimize the interference of a potential secondary structure dependent mechanism. The changes introduced yielded the same *in silico* folding pattern as the wild type sequence. Primer extension analysis clearly indicated a decreased cutting signal from mRNA containing this modified motif, as compared to mRNA containing the native sequence (Fig. 3.2A, right panel). In comparison to the wild type CSC-box (AGUAAA), the mutated sequence (AGUCCC) exhibited a 2 to 5 fold decrease in cleavage signal compared to the wild type sequence (left), but the cleavage was not completely inhibited (Fig. 3.2A, left panel).

Fig. 3.2 Primer extension analysis of the CSC-box inserted in the *lacZ'* fragment (next page). Specific fragments (50bp) derived from *cspA1/A2* sequence, contained either wild type CSC-box sequence AGUAAA (left) or the mutated CSC-box AGUCCC (right, changes underlined) were inserted in frame with *lacZ'* in pBSK(K)1 plasmid and analysed in *Y. enterocolitica* after cold-shock. mRNA was constantly induced with IPTG. Sampling times are given in minutes above. In the middle of each panel, sequence of pKD1-15 plasmid is shown like a ladder for band detection. +1 signal and detected cutting signals are marked with side arrows. Panel **A**: Primer extension analysis after a cold-shock from 30°C to 10°C. CT, control (CT) at 30°C before cold-shock. Panel **B**: Primer extension analysis of the wild type CSC-box sequence and mutated *lacZ'* mRNA after addition of rifampicin. Ten minutes after cold-shock a sample was taken (= 0 min) and rifampicin was added to block transcription. Samples after addition of rifampicin were taken at the time points indicated. As expected, the +1 signal disappears.


A
AGUAAA
CT 2 30 45 60 C T/U A G
AGUCCC
C T/U A G CT 2 30 45 60
+1
cut
B
AGUAAA
0 15 30 50 70 90 C T/U A G
AGUCCC
C T/U A G 0 15 30 50 70 90

To determine whether the mutated AGUCCC cut site decreases the decay rate in comparison to the wild type sequence AGUAAA, rifampicin was added 10 min after cold shock. The inhibition of transcription was followed by monitoring the reduction of the +1 signal (Fig. 3.2B). Calculation of the band intensities of the +1 sites revealed only a slightly higher decay rate of the *lacZ* mRNA containing AGUAAA in comparison to the altered AGUCCC. This indicates that not only the AGUAAA motif, but also the flanking sequences play a role in the cleavage process.

3. 3 Occurrence of the AGUAAA cutting site in *cspB* of *E. coli*

The *cspB* mRNA of *E. coli* is cold inducible and contains two AGUAAA motifs, 24 and 79 nucleotides downstream of the start of the coding sequence. To test, whether the AGUAAA motif has a function in bacteria other than *Y. enterocolitica*, primer extension analysis of RNA isolated from *E. coli* cells shifted from 37°C to 15°C was performed with a probe specific for *cspB* mRNA (cspB-3). Using this probe, we identified four RNA species that are presumably generated by endonucleolytic cutting of the primary transcript (Fig. 3.3). The first cutting occurs within the first AGUAAA motif (AGUAAA, underlined). The second and third cuts were found two and eight nucleotides downstream of the first cutting site, respectively. Finally, the fourth cutting occurred within the second AGUAAA motif, (AGUAAA, underlined). Thus, our results appear to demonstrate the importance of the AGUAAA motif for the control of mRNA stability of cold inducible *csp* mRNA not only in *Y. enterocolitica,* but also in *E. coli*.

3. 4 Cleveage of AGUAAA motif in *E. coli* RNase E mutants

Degradation of mRNA in bacteria is mainly studied in *E. coli* and *B.subtilis*. In lack of specific mutants for mRNA decay in *Y. enterocolitica,* construct pKD1-15 (with wild-type sequence AGUAAA for specific degradation) was analyzed in *E. coli* MG1693 strain (wild type) and in its specific mutant strains SK5704 and SK5665. Figure 3.4A shows the decay pattern of the mRNA from pKD1-15 construct in RNase E mutant (strain SK5665) and in the triple mutant on Fig. 3.4B (strain

SK5704 containing mutations for RNase E, PNPase and RNase II) previously shown to be involved in mRNA degradation.

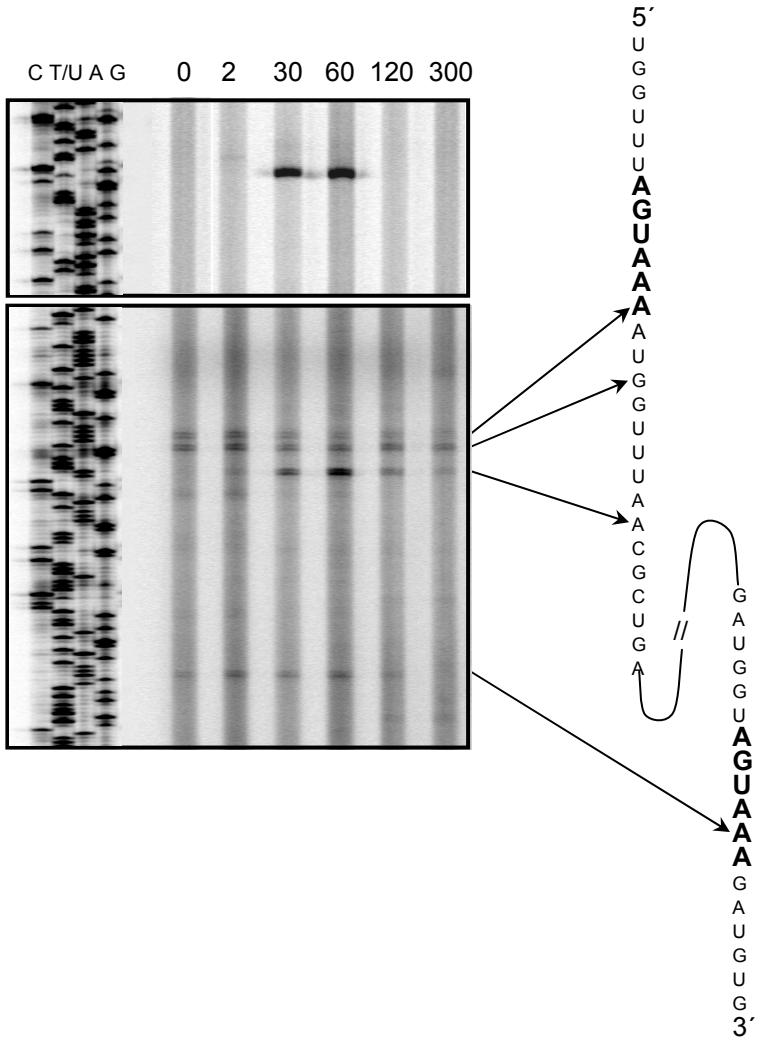


Fig. 3.3 Primer extension analysis of *E. coli cspB* mRNA. Cells were cold shocked from 37°C to 15°C and samples were taken at the time points indicated in minutes. The sequencing ladder was obtained by sequencing specific PCR fragment for *cspB* gene in *E. coli* with the same primer used for primer extension experiemnts (cspB-3). Arrows localize the cut sites in the corresponding *cspB* sequence. Note that one cutting is visible within the first AGUAAA motif and two cutting sites in close vicinity downstream of this motif. One cutting site can be seen in the second AGUAAA motif.

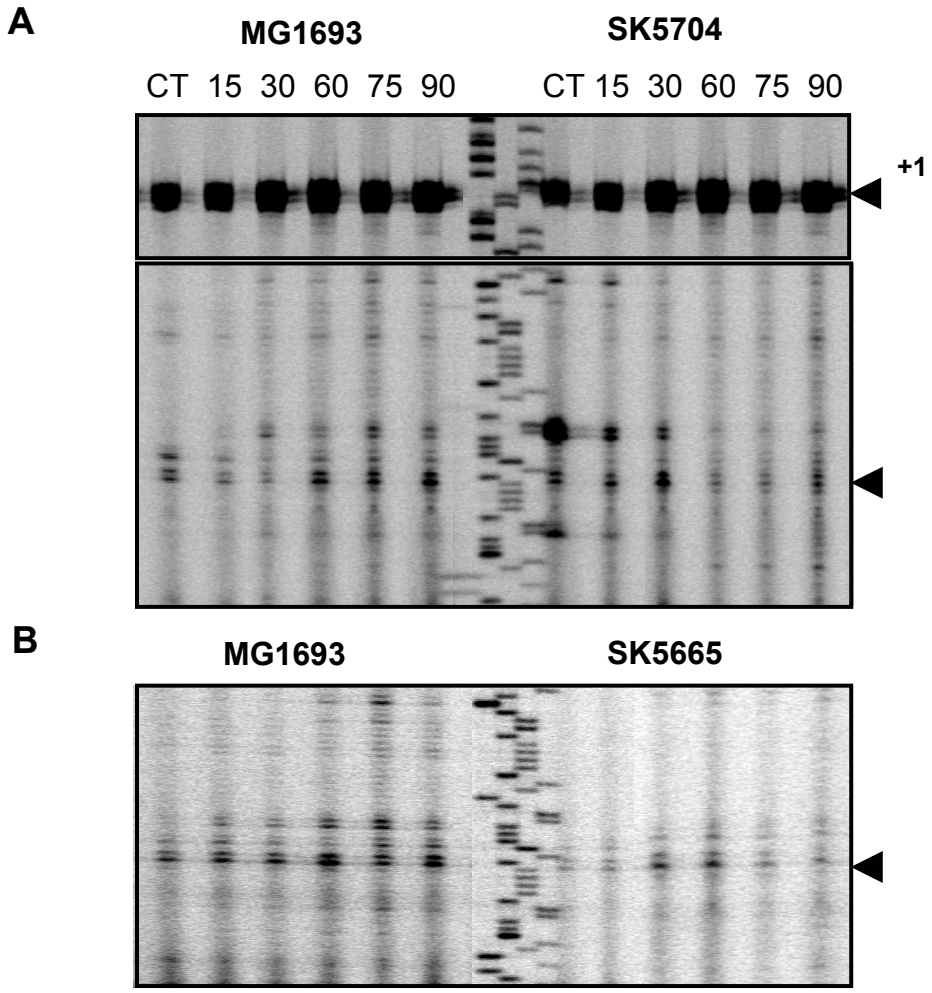


Fig. 3.4 Primer extension analysis of the CSC-box in the RNase E mutants. Inserted 50 bp - fragments containing the wild type CSC-box sequence AGUAAA were analysed in *E. coli* strains after heat shock at 42°C. mRNA was constantly induced with IPTG. Samples were taken at the time points indicated above. In the middle of each panel, sequence of pKD1-15 plasmid is shown like a ladder for band detection. +1 signal and detected cutting signals are marked with side arrows. Panel **A**: Primer extension analysis after heat shock from 30°C to 42°C. CT, control at 30°C before heat shock. CSC-box sequence inserted in *lacZ'* mRNA was analysed in *E. coli* wild type (MG1693) and triple (RNase E, RNase II, and PNPase) temperature sensitive mutant (SK5704). Panel **B**: Primer extension analysis of the wild type CSC-box sequence after heat shock from 30°C to 42°C in *E. coli* wild type (MG1693) and RNase E temperature sensitive mutant (SK5665).

Cultures were incubated at 30°C to an OD_{600} 0,5 and samples were taken at different time points after heat shock at 42°C, because *rne* (encoding RNase E) and *rnb* (encoding RNase II) are temperature sensitive mutant alleles. Results presented on Fig. 3.4A show a difference in mRNA decay between wild type *E. coli* MG1693, and mutant strain SK5704, even at 30°C. After a heat shock at 42°C RNA degradation was efficient in wild type, but less efficient in mutant strain. After heat shock the signal intensity for degradation product in region of specific sequence AGUAAA at 90 min and 120 min was weaker in mutant strain SK5704. The same result was obtained with mutant strain for RNase E (SK5665). Decrease in decay product was observed 75 min and 90 min after heat shock in RNase E mutant strain.

3. 5 The AGUAAA motif is important but not sufficient for specific cleavage by RNase E

To further test the influence of the sequences adjacent to the AGUAAA motif on the cleavage of *cspA1/A2* mRNA, two short RNA oligomers containing the AGUAAA motif were assayed *in vitro* using the catalytic domain of RNase E (residues 1 – 498). The first RNA oligomer (16mer-cut2) was derived from the *cspA1/A2* mRNA sequence around the “cutting 2” site (see Fig. 3.1) and contained 16 bases of an extended consensus sequence. The second oligomer (16mer-cut3) was derived from the site “cut 3” and was chosen for two reasons: its surrounding sequence showed no homology to the extended consensus sequence, and it has an exceptional cutting site within AGUAAA motif (A underlined) found in *cspA1/A2* of *Y. enterocolitica*. After radiolabelling, both RNA oligomers were incubated 10 min and 60 min with the catalytic domain of RNase E from *E. coli*. Surprisingly, only the first RNA oligomer (16mer-cut2) was cleaved at the first A of the CSC box (AGUAAA, underlined). The second RNA oligomer (16mer-cut3) was relatively resistant to the nucleolytic activity of the enzyme (Fig. 3.5). This result can be explained by the assumption that the AGUAAA motif is necessary, but not always sufficient to provide a high efficiency cleavage in the absence of the flanking secondary structure region.

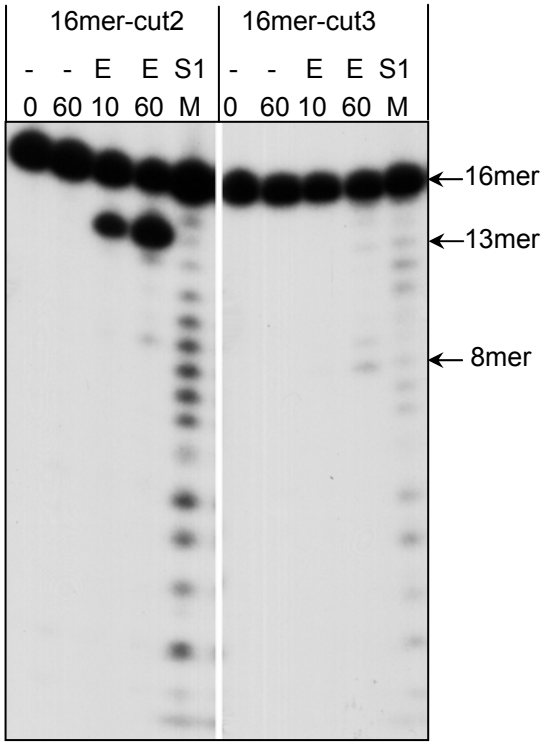


Fig. 3.5 *In vitro* RNA decay experiments. Short RNA oligonucleotides were derived from *cspA1/A2* sequence. The sequence of the 16mer-cut2 is 5´-UUU**AGUAAA**AUGGUUU-3´, and for the 16mer-cut3 is 5´-CCA**AGUAAA**UUAAGCA-3´, the CSC boxes are marked in bold print. RNA oligonucleotides were incubated with buffer only (indicated by -) for 0 and 60 min, with the catalytic RNase E domain (indicated by E) from *E. coli* for 10 and 60 min, and with S1-nuclease (indicated with S1), to obtain a marker (M). Individual length of some marker bands are indicated.

To prove that RNase E is responsible for specific cleavage that is dependent on the flanking sequences, total mRNA was isolated from *Y. enterocolitica* cells 30 min and 60 min after cold shock, and subjected to *in vitro* analysis with RNase E and RNase G enzymes (Fig. 3.6). Results showed that neither RNase E nor RNase G seems to be responsible for such a specific cutting. RNase G is not cutting isolated total mRNA at all, and RNase E has two additional cutting sites downstream from proposed CSC box. It can be proposed that additional RNase in *Y. enterocolitica* is responsible for such specific cutting, or more likely some

unknown factor contribute to this precise cut, directing RNase E to specific cutting site(s) only on cold.

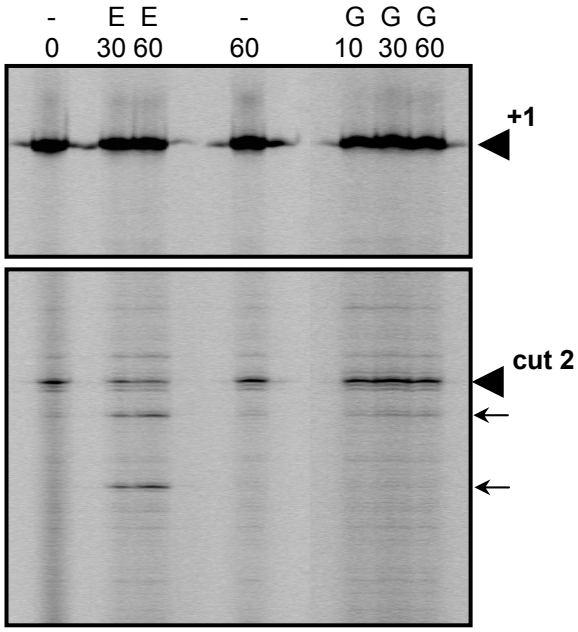


Fig. 3.6 *In vitro* RNA decay experiments with total mRNA isolated 60 min after cold shock. Total mRNA was incubated with buffer only (indicated by -) for 0 and 60 min, with the purified *E. coli* RNA degradosome (indicated by E) for 30 and 60 min, and with *E. coli* RNase G (indicated by G) for 10, 30 and 60 min. Original cutting site obsereved before on Fig. 3.1B is idicated with thick arrow like ‚cut2'. Additional not specific cutting sites observed after incubation with RNA degradosome are labeled with thin arrows.

3. 6 Sequencing and sequence analysis of genes involved in mRNA degradation in *Y. enterocolitica*

In *E. coli* the multiprotein complex for mRNA decay called „degradosome" consist of several protein components. The key enzyme for degradation as well as for the assembly of the degradosome is RNase E. It was noted that RNase E copurifying with PNPase and additional proteins. Amino terminal sequencing of some additional proteins identified the glycolytic enzyme enolase and RNA helicase RhlB as components of the complex (Steege 2000). Identification of additional proteins are necessary for further analysis of degradosome complex and according

to that we decided to sequence the genes proposed to be involved in mRNA degradation in *Y. enterocolitica.* By combining PCR, inverse PCR and primer walking methods sequencing of RhlB helicase (*rhlB* gene), enolase (*eno* gene) and RNase III (*rnc* gene) was possible. Sequences are presented in Appendix. RNase E (*rne* gene) was partially sequenced. PNPase and DeaD helicase were also sequenced and analyzed in more details explained later in the result section. Further work was mainly focused on PNPase and DeaD helicase because this region already showed very important characteristics for cold adaptation in *E. coli* and *Y. enterocolitica*.

3. 7 Sequencing and sequence analysis of *deaD* gene from *Y. enterocolitica*

Sequencing of *deaD* gene from *Y. enterocolitica* was done by combining PCR, inverse PCR and primer walking methods. Sequence analysis indicated that 6253bp sequenced fragment contain open reading frames (ORFs) with similarities to PNPase, NLP1 (hypothetical lipoprotein) and ORF with high similarity to DEAD box RNA helicases. This ORFs are tentatively designated with the genes *pnp, nlp1* and *deaD* (Fig. 3.7).

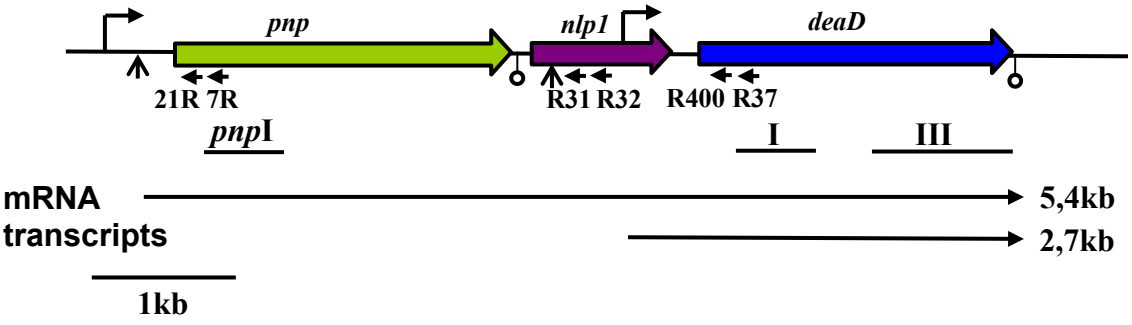


Fig. 3.7 Genetic and transcriptional organization of the *pnp-nlp1-deaD* locus. The 6253bp fragment of *Y. enterocolitica* strain encompasses three genes. Open arrows represent the position and extent of the three genes. The bent arrows upstream of *pnp* and *deaD* genes indicate detected transcriptional start sites in the *pnp-nlp1-deaD* operon and the direction of transcription. The vertical arrows indicate detected RNase III cutting sites by primer extension analysis. Proposed transcription termination sites are marked with hanging balls. Locations of the primers (complete sequences are presented in Tab. 2.3) used in primer extension experiments are depicted by arrow heads below the *pnp*, *nlp1* and *deaD* genes. The bars below the map (I, III and *pnp*I) represents the PCR-amplified DNA fragments used as probes in Northern blot experiments. Horizontal arrows below the map indicates the two mRNA transcripts detected by Northern analysis on Fig. 3.10 (the 5,4kb is *pnp-nlp1-deaD* polycistronic transcript and the 2,7kb is *deaD* transcript).

Analyses of the sequence flanking the ATG initiator methionine codon of the *deaD* gene show homology with the putative regulatory transcriptional and translational elements responsible for activation of cold shock inducible genes (Fig. 3.8).

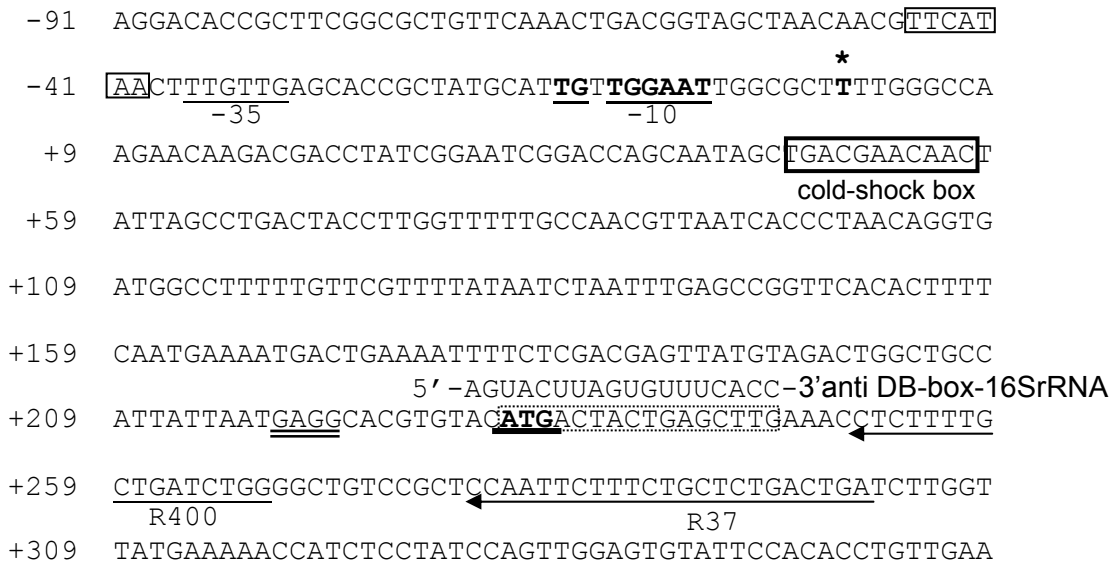


Fig. 3.8 Sequence analysis of putative transcriptional-translational elements of *deaD* gene. Shown is the DNA sequence surrounding the ATG initiator methionine codon presented in bold. Numbering reflects the transcription start site determined by primer extension and taken to be +1 (indicated bold and by an asterisk). Putative regulatory elements are indicated as follows: boldface and underlined, extended -10 region; underlined, -35 region; outlined box, AT-rich upstream element; thickly outlined box, cold shock box; double underlining, putative RBS; and doted box, possible downstream box (DB). Position of primers used in primer extension reactions is marked with arrows. 16S rRNA sequence part named anti-downstream box is placed according to the sequence for DB for comparison.

The RNA helicase protein encoded by *deaD* gene is characterized by eight motifs, including DEAD (Asp-Glu-Ala-Asp) tetra-peptide, which is characteristic of this family of helicases. The complete sequence of the putative helicase is 1986bp long, and encodes a 662 amino acid polypeptide with a predicted molecular mass of 73kDa and a pI of 10,15. DeaD from *Y. enterocolitica* is most closely related to the *E. coli* characterized RNA helicase, CsdA, having 81 % identity on a protein level. DeaD from *Y. enterocolitica* is related to other four DEAD box RNA helicase sequences from *E. coli* with 61 % to 81 % identities. *Y. pestis* and *Y. enterocolitica*

sequence database from Sanger institute showed 92 % to 98 % identity on a protein level, respectively.

3. 8 C-terminus of DeaD helicase has a high homology with DbpA helicase

DEAD proteins share a set of eight conserved amino acid sequence motifs (Fig. 3.9). Variable extensions found to either side of these conserved motifs are thought to facilitate the unique functions of individual DEAD proteins. Database searches revealed that DeaD protein in *Y. enterocolitica* has a C-terminal high homologie to the C-terminus of DbpA protein in *E. coli* (Fig. 3.9) (Nicol and Fuller-Pace 1995) and YxiN in *B. subtilis* (Kossen and Uhlenbeck 1999).

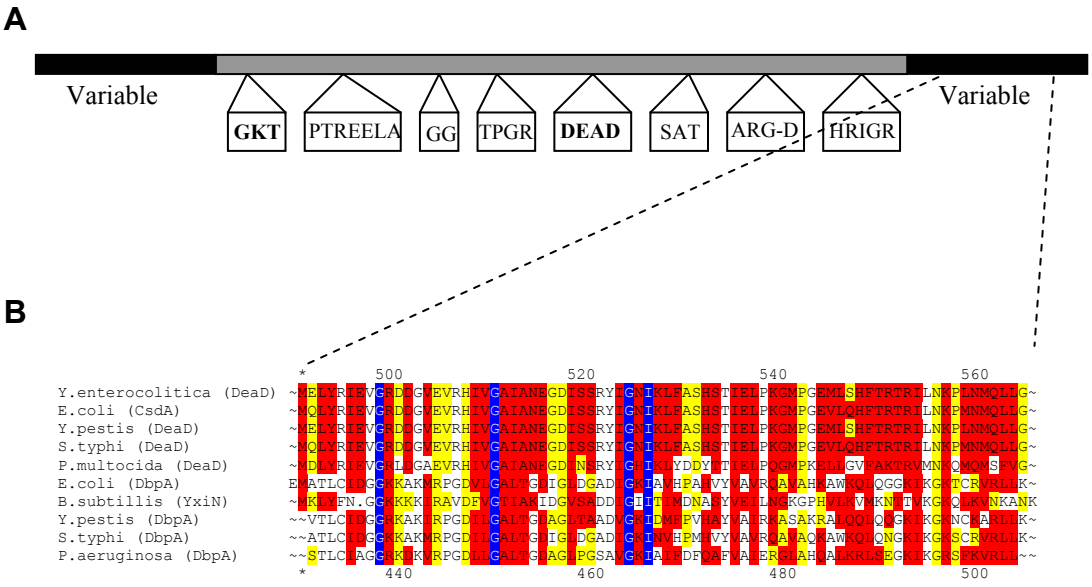


Fig. 3.9 Schematic presentation of motifs conserved among DEAD proteins. Panel **A)** An idealized DEAD protein with eight conserved amino acid sequence motifs and variable N- and C-terminal extensions is presented. Motifs involved in binding and hydrolysis of ATP are presented in bold. Panel **B)** Multiple sequence alignment of the C-terminal domains of DeaD, DbpA and eight putative homologues. Alignment was done by ClustalW program. Amino acid residues are represented as a single letter codes and sequences with a low degree of conservation or gaps (-). Region around 500 amino acid (C-terminus) is high hydrophilic region of protein determined by Kyte-Doolittle plot analyses from HUSAR program (Heidelberg, Germany) and has a high homology with specific region for binding a 23S rRNA in DbpA protein. Note the conservation of four basic amino acids, where the precise position of conserved glycines is the most important for this specific domain involved in binding of 23S rRNA specific loop. Conserved amino acids groupings used in this alignment are: 100 % identity, blue (black), 50 % identity with strong amino acid grouping, red (dark gray) and 50 % identity with weak amino acid grouping, yellow (light gray).

The other three described helicases from *E. coli* do not contain this specific C-terminal region. Only CsdA from *E. coli* and DeaD from *Y. enterocolitica* have this high homology in the C-terminus with DbpA helicase. DbpA protein is unique among the known DEAD proteins in that its ATPase activity is specifically stimulated by bacterial 23S rRNA. A C-terminus extending 120 amino acids beyond the last characteristic DEAD motif was considered most likely to be responsible for the RNA specificity of DbpA whose activity depends upon the presence of hairpin 92 of 23S rRNA and precisely positioned substrate helices (Nicol and Fuller-Pace 1995; Diges and Uhlenbeck 2001). Taken together it is possible that the C-terminal regions of DeaD and CsdA protein are important for stimulation of ATPase activity by certain mRNA secondary structures, during cold shock.

3. 9 Induction of *deaD* gene expression during the acclimation phase

We examined the transcriptional profile of the *deaD* gene in the cold acclimation phase by Northern blot analysis. The RNA extracted at different times from samples of a *Y. enterocolitica* culture (grown at 30°C and than shifted to 10°C) was hybridized with probe III (Fig. 3.7). A transient modification of the *deaD* transcript profile during the first hour after cold-shock (Fig. 3.10) was observed. The intensity of the main signal detectable at 30°C (2,7kb; lane 0) progressively increased from 30 min to 120 min after cold shock, before returning to a preshift level after 150 min incubation at 10°C. A similar pattern was seen for a longer RNA transcript (5,4kb) detected with the same probe. The cumulative intensity of the 2,7kb and 5,4kb bands at 30 min after cold-shock was 2,5 fold and 6 fold higher than at 30°C, respectively, as measured and calculated by using ImageMaster 1D Elite version 3.00 (Amersham Pharmacia Biotech, Heidelberg, Germany). The transcripts of 2,7kb and 5,4kb detected by the *deaD* probe III were mapped by Northern blot hybridization also with the *deaD* probe I (Fig. 3.7). By hybridizing with the probe from the *pnp* coding region (data not shown) we confirmed that the 5,4kb mRNA spans from *pnp* to *deaD* and presents polycistronic mRNA which was readily detectable at 30°C and induced at 10°C.

The transcription termination site was characterized downstream of *pnp* gene (∆G° of –15.3kcal) (Goverde, Huis in't Veld et al. 1998). Similar transcription termination site can be proposed at 3' UTR region of the *deaD* gene. The putative 25-nucleotide stem-loop (hairpin) secondary structure was found 28 nucleotides downstream of the translational stop codon for *deaD* (Fig. 3.7) and characterized like Rho-independent transcription termination site (Hajnsdorf, Carpousis et al. 1994). This potential transcriptional terminator consists of 10bp stem and 6bp loop structure followed by a string of three T residues with ∆G° of –12.6kcal/mol. Detection of the 2,7kb band in Northern blot analysis was in agreement with this predicted termination sites. According to this finding it was proposed that *deaD* gene has its own promoter and independent transcription from *pnp* during cold shock.

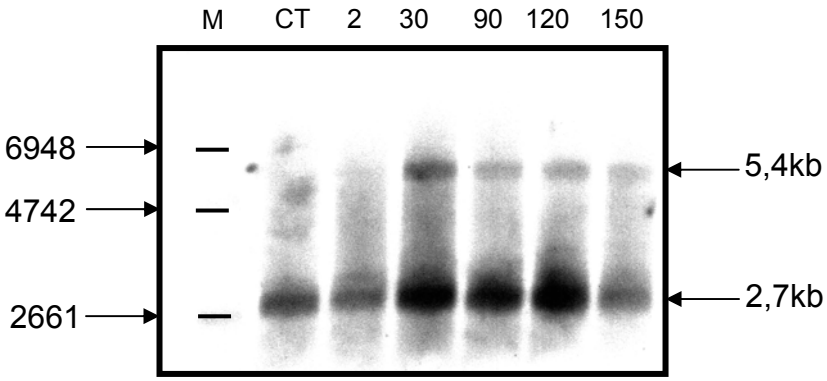


Fig. 3.10 Northern analysis of *csdA* expression. Total RNA was extracted from *Y. enterocolitica* cells grown to mid-log phase at 30°C and than shifted to 10°C. Samples were taken just before cold-shock (0 point) and at different time points after cold-shock (indicated on the top of the figure). Northern blot was performed by using probe III (see Fig. 3.8). Arrows mark the position of detected 5,4kb *pnp-nlp1-deaD* polycistronic transcript and the 2,7kb *deaD* transcript. The positions of DIG-labelled RNA marker are shown to the left.

3. 10 Determination of transcriptional signals in the *pnp-nlp1-deaD* locus by primer extension analysis

Two regions were analyzed for promoter activity and detection of transcriptional start site in front of *pnp* gene and in front of *deaD* gene, by using six different primers labelled on Fig. 3.7.

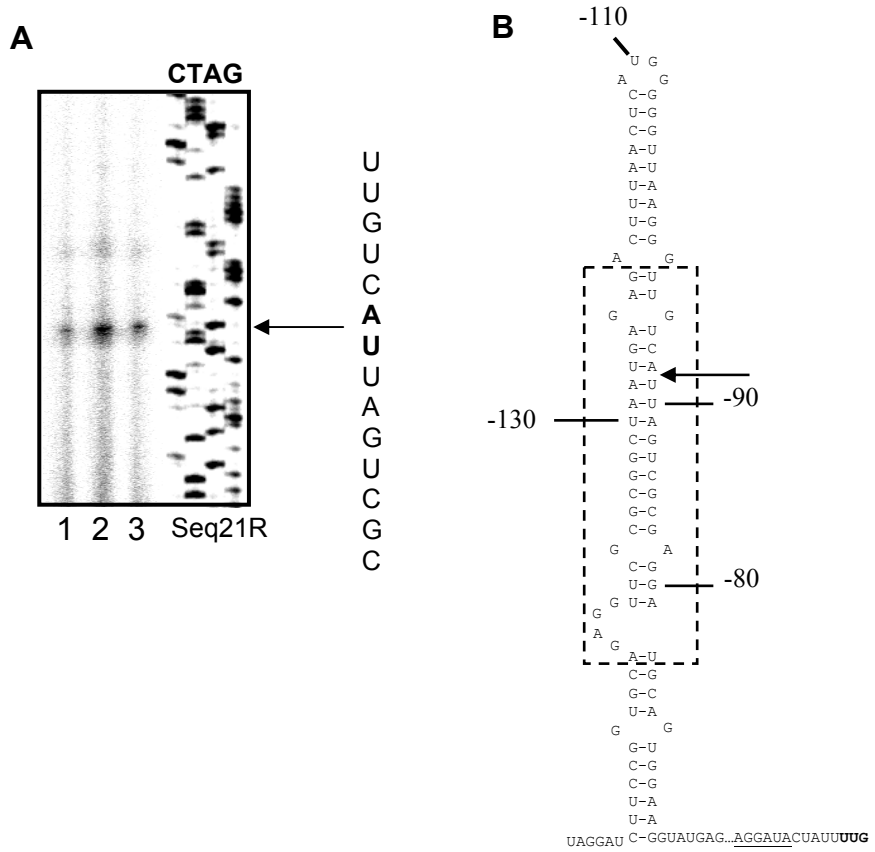


Fig. 3.11 Panel **A: Primer extension analysis of the 5' end of the 5,4kb *pnp-nlp1-deaD* polycistronic transcript.** Figure shows a primer extension experiment performed on RNA extracted from *Y. enterocolitica* grown at 30°C until mid log phase (lane 1) and from the samples 45 min and 60 min after cold-shock treatment (lane 2 and 3), with primer 21R (see Tab. 2.3 and Fig. 3.7 for primers). Lanes C, T, A, and G show the sequencing ladder generated with the same primer 21R. The right arrow mark the end-point of the extended product equal with position -91 marked with arrow at part B. Panel **B: Predicted secondary structure in the *pnp* leader transcript**. The most thermodynamically stable structure of the 5' end *pnp* mRNA leader carrying the RNase III site was predicted by the *mfold* computer program (Mathews, Sabina et al. 1999). Nucleotides framed by dotted line are conserved in *E. coli*, *Y. enterocolitica* and *Photorhabdus* sp. Arrow points to the determined 5' end by primer extension analysis in *Y. enterocolitica.* This determined 5' end is in correlation with downstream RNase III cleavage site previously shown by S1 mapping and primer extensions in *E. coli* (Jarrige, Mathy et al. 2001).

The primer extension product, generated by primers specific for *pnp* gene, was detected as an AU doublet (Fig. 3.11A). The proximal A residue is located 91 nucleotides upstream of the *pnp* translational start codon. Intensity of the primer extension product increased in cold-shock induced cells (Fig. 3.11A, lines 2 and 3). A long irregular stem-loop is predicted to form in the *pnp* leader carrying an RNase III site (Fig. 3.11B) and this region is highly conserved in *E. coli*, *Y. enterocolitica* and *Photorhabdus* sp.

The primer extension product generated by primers specific for *deaD* gene was detected as a T residue, located 232 nucleotides upstream of the *deaD* proposed translational start codon. An increase of primer extension product after 60 min on cold was observed (Fig. 3.12, line 2), in agreement with the Northern blot analyses (Fig. 3.10). Potential secondary structure by the *mfold* program (Zuker 1989) for RNase III processing site was not found in this region.

By using a primers specific for *deaD* gene it was possible to detect a primer extension product as a C residue, located 846 nucleotides upstream of the proposed ATG transnational start signal for *deaD* gene. This signal was proved with two additional primers from the *nlp1* coding region, and was clearly induced after incubation of cells for 60 min at 10°C (Fig. 3.13, line 2). This C residue was in a specific stem-loop structure predicted by the *mfold* program (Zuker 1989). The prediction pointed one more possible site for RNase III processing.

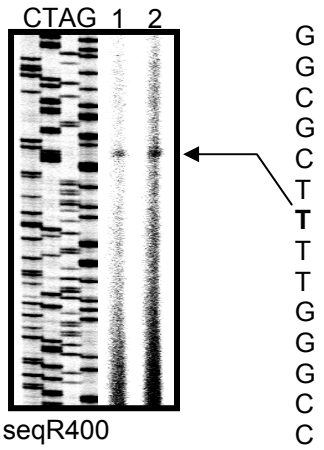


Fig. 3.12 Determination of transcription start site for *deaD* gene. Primer extension experiment performed with RNA extracted from *Y. enterocolitica* cells grown at 30°C up to mid-log phase (lane 1) or 60 min after cold shock at 10°C (lane 2). Lanes C, T, A, G are sequencing reactions. Primer extension reactions and sequencing reaction were performed with the same oligonucleotide R400. An arrow indicates 5' end product corresponding to the T residue taken to be the start of transcription for *deaD* gene.

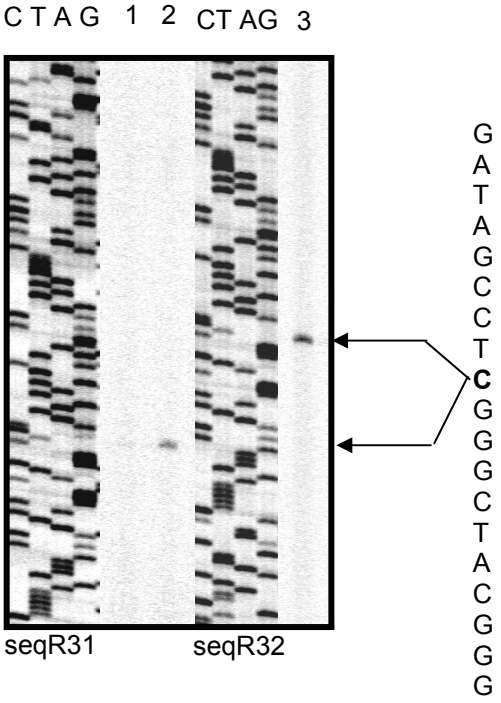


Fig. 3.13 Determination of transcription start site for *nlpI* gene. Primer extension experiment performed with RNA extracted from *Y. enterocolitica* cells grown at 30°C to mid-log phase (lane 1) or 60 min after cold shock at 10°C (lane 2 and 3). Lanes C, T, A, G are sequencing reactions performed with oligonucleotide R31 and lane 1 and 2 the primer extension product with the same R31 primer. The right part of the figure with C, T, A, G lanes and lane 3 presentes reactions with R32 oligonucleotide. A double arrow indicates primer extension product corresponding to the C residue taken to be site for possible RNase III processing.

3. 11 Identification of promoter activity by transcriptional fusions with *egfp* gene

The results described above pointed to the existence of at least three independent primer extension products in the *pnp-nlp1-deaD* region. To determine whether the cold-shock induced transcription profile detected during Northern and Primer-extension analysis could be related to different promoter activities, different transcriptional fusions with Enhanced Green Fluorescent Protein (EGFP) were generated as described in Materials and Methods.

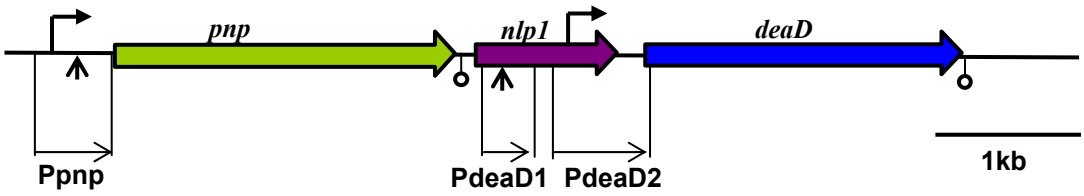


Fig. 3.14 Regions used for subcloning in pEGFP promoter probe vector (marked with thin arrows). Genetic organization of *pnp-nlp1-deaD* locus is marked the same as on Fig. 3.8.

Measurements of EGFP levels derived from the various constructs in the *Y. enterocolitica* cells at 30°C were measured and presented in Tab. 3.1. With respect to the influence of different subcloned promoter regions, it can be seen that *lacZ* promoter is the strongest promoter between those analyzed at 30°C, what was actually expected as *lacZ* is one of the most strongest promoter known. During mid-exponential growth phase (OD_{600} 0,5) the activity of *deaD*2 promoter and *lacZ* promoter was almost the same. After incubation of cells to an early stationary phase (OD_{600} 1,4), the activity of *deaD*2 promoter reached 1568 RFU, and was 2,5 times less active compared to the activity of the *lacZ* promoter (see Fig. 3.13A). The *pnp* promoter was even weaker and showed a 4,5 fold decrease in activity than *lacZ* promoter in the same growth phase. The *deaD1* analyzed region was assumed to be completely inactive for promoter activity.

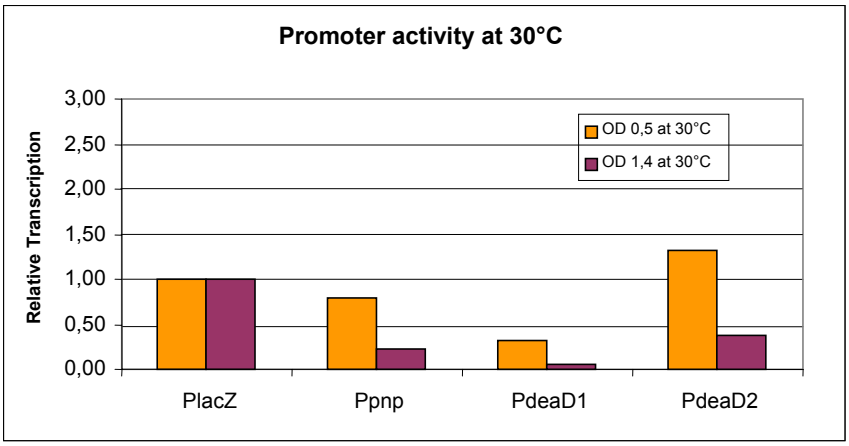
Table 3.1 Quantification of promoter activity by measuring of EGFP fluorescence

RFU[a]	OD_{600}	*lacZp*	*pnpp*	$deaDp_1$	$deaDp_2$
at 30°C	0.5	833±288	661±119	273±236	1105±320
at 30°C	1.4	4065±257	938±259	199±180	1568±280
at 30°C-before cold shock	0.5	845±320	712±105	188±169	1081±350
at 10°C-4h after cold shock	0.8	635±250	611±198	422±210	1593±310
at 10°C-20h after cold shock	1.8	43±163	673±221	13±255	2052±325

[a] (**RFU**) - Relative Fluorescence Units - Fluorescence activity of EGFP expression presents ratio of fluorescence intensity to cell density (520nm/405nm) substracted with background activity of the cells with native pEGFP promoter probe vector. The values represent the average of three independent experiments.

To examine weather *pnp* or *deaD* promoters were low temperature inducible in *Y. enterocolitica*, EGFP levels of the transcriptional fusions were measured, after cold shock. For *pnp-egfp* fusion in Tab. 3.1 was shown that the level at 30°C was nearly identical to that observed at 10°C after 4h and 20h incubation, suggesting that expression of the *pnp-egfp* fusion at low temperature was not temperature inducible. This is in correlation with recently published data that *pnp* promoter is not low temperature inducible in *E. coli* (Mathy, Jarrige et al. 2001).

A



B

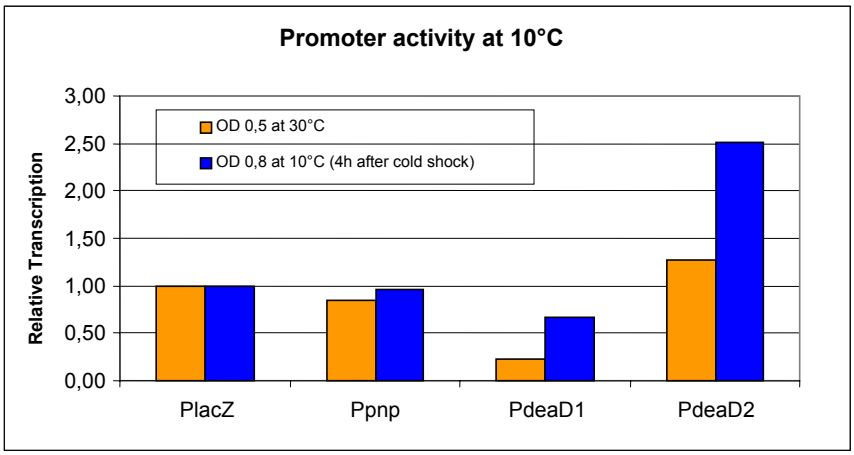


Fig 3.15 Promoter activity determined by measuring of EGFP fluorescence in *Y. enterocolitica* cells with different transcriptional *egfp* fusions, during growth at 30°C (Panel A) and after a cold-shock at 10°C (Panel B). RFU activities of P*pnp*, P*deaD1* and P*deaD2* promoters from Tab. 3.1 were normalized to the activity of transcriptional fusion with P*lacZ* promoter and data were shown as transcription relative to cells with native pEGFP. PlacZ – contains *lacZ* promoter; Ppnp – contains *pnp* promoter; PdeaD1 – contains *deaD1* region (see Fig. 3.12) analyzed for promoter activity and PdeaD2 – contains *deaD2* promoter, in front of *egfp* reporter gene.

The activity of *deaD2-egfp* fusion reached 1593 RFU at 10°C four hours after cold shock. This was a 2,5 fold increase in activity compared to the activity of *lacZ* promoter at 10°C under the same conditions (Fig. 3.15B). Expression of EGFP from

lacZ promoter decreased during incubation on cold, and was almost absent after 20h incubation at 10°C while *deaD2* promoter had very high activity under this condition (Tab. 3.1). The activity of *deaD*2 promoter reached 2052 RFU 20 hours after cold-shock and had 2 fold increase in activity compared to the activity of P*deaD* promoter at 30°C, before cold shock.

4 DISCUSSION

4.1 The CSC box occurs in a variety of bacteria

Northern blot data identified the existance of multiple mRNA species originating from the bicistronic *cspA1/A2* transcript when the cells restart growth (Neuhaus, Rapposch et al. 2000). Primer extension experiments demonstrated that the 5´end of *cspA1/A2* mRNA decay products in *Y. enterocolitica* are formed by cleavage within the core sequence AGUAAA (Fig. 3.1 and 3.2). These findings lead us to propose a hypothesis, which is centered around specific *cspA* mRNA cleavage and degradation. The consensus cleavage sequence AGUAAA identified in this study was termed **C**old **S**hock **C**ut box (CSC-box).

In a systematic database search, 69 complete CSP sequences from different bacterial species were found. The CSC-boxes identified in *Y. enterocolitica* were also found in a large number of other bacteria. An alignment performed by using ClustalW (Baylor College of Medicine, 1999) shows that CSC-box homologues mainly belong to 4 different groups: sequence (A)GUAAA in 46 % cases, (A)GUUAA in 20 %, (C)GUGAA in 17 %, and YGUCAA (Y being C or T) in 9 % was found. In 5 cases (8 %) no homology could be detected. The nucleotides appearing in brackets occur in most (≥ 50 %) but not in all cases. At least 66 % of the sequences examined (including a diverse range of genera, e.g., *Bacillus*, *Escherichia*, *Thermotoga*, and others) share a CSC-box homologoue to the consensus sequence (A)GU(A/U)AA. Table 4.1 lists a sample of CSP homologues that share the sequences AGUAAA or AGUUAA. The similarity of the CSC motif in a variety of taxonomically unrelated bacterial species (Tab. 4. 1) suggests an important biological function for this motif in bacteria in general. Nevertheless, sequence homology as such can be quite misleading. Otherwise, one would have expected that this sequence diverge extensively between Gram negative and Gram positive bacteria. Furthermore, we examined the cleavage of the *csp*B mRNA in *E. coli,* and found a cleavage pattern occurring in close vicinity to the AGUAAA motif (Fig. 3.3). Although this result supports a similar function in *E. coli*, cleavage did not occur exactly at the same site as in *Y. enterocolitica*. If the CSC box is part of a general mechanism of CspA down regulation, a hypothesis requiring further experimental substantiation, the cleavage sites may vary within different species.

Tab. 4.1 Bacterial *cspA* sequences which contain the CSC-box AGUAAA or AGUUAA

CSC-box or homologue	bacterium	MCSP homologue[a]	database accession no.
AGUAAA	*Bacillus caldolyticus*	B	X73373
	Bacillus cereus	A	X93039
	Bacillus cereus	C	X93041
	Bacillus cereus	D	X93042
	Bacillus cereus	E	X93043
	Bacillus stearothermophilus	B	X73375
	Bacillus subtilis	B	X59715
	Bacillus subtilis	D	L77246
	Escherichia coli	B	L28429
	Escherichia coli	G	AF003591
	Lactobacillus plantarum	L	Y08940
	Lactococcus lactis	A	Y17215
	Lactococcus lactis	D	Y17216
	Listeria monocytogenes	L	X91789
	Salmonella typhimurium	B	Y11932
	Shewanella violacea	G	AB022716
	Staphylococcus aureus	B	AF003592
	Yersinia enterocolitica	A1	U82821
	Yersinia enterocolitica	A2	U82821
	Yersinia pestis	G	Z97978
AGUUAA	*Aquifex aeolicus*	C	AE000733/AE000657
	Bacillus cereus	B	X93040
	Bacillus subtilis	C	U92976
	Listeria monocytogenes	Lb	U90213
	Rickettsia prowazekii	A	AJ235272/AJ235269
	Staphylococcus aureus	C	AF003593
	Yersinia enterocolitica	B	AF070484

[a] The names of the homologues were taken from database and do not necessarily indicate that the CSPs named with the same letter belong to the same functional group.

4. 2 Interaction between the downstream box (DB) and the CSC-box?

The downstream box (DB) is a sequence element that enhances translation of several bacterial and phage mRNAs. It has been proposed that the DB enhances translation by transient base pairing to bases 1469-1483 of 16S rRNA, the so-called anti-DB, during the initiation phase of translation (O'Connor, Asai et al. 1999). In

some cold - inducible mRNAs, a DB can be found immediately 3´ to the start codon ATG (Mitta, Fang et al. 1997; Etchegaray and Inouye 1999; 1999). This box has been reported to be essential in cold-shock induction of *cspA* and *cspB* in *E. coli*. However Blasi, O'Connor et al. (1999), and also O'Connor, Asai et al. (1999), have proposed that the DB may act to directly effect mRNA stability. In *Y. enterocolitica* two CSC-boxes (i.e. “cut 2”) are located directly within the DB sequence. This finding supports the hypothesis that mRNA stability is involved in the regulation of CSP’s synthesis under cold shock, rather than base pairing between DB and anti-DB on 16S rRNA (O'Connor, Asai et al. 1999; Moll, Huber et al. 2001).

4. 3 Downregulation of *cspA* mRNA is vital for low temperature growth

In *Y. enterocolitica* and other bacteria, CSPs are important in allowing cells to overcome the impediment of ribosomal stalling resulting from a temperature downshift. In order for a bacterium to synthesize CspA after cold shock, *cspA* mRNA needs to have a robust translatability. This feature is determined at the step of initiation via ribosomes (Xia, Etchegaray et al. 2001), leading to a preferential synthesis of CspA and some other cold-shock proteins upon temperature downshift. However, during the ongoing process of cold adaptation, the level of the CSP mRNAs needs to be downregulated, otherwise the ribosomes will stay trapped by this mRNA and translation of bulk mRNA will be inhibited. It has been shown that an increased level of *cspA* mRNA is detrimental for *E. coli,* and that overexpression of the 5´ untranslated region of *cspA* leads to inhibition of growth (Jiang, Fang et al. 1996). Similarly, if the decay of CSP mRNA fragments is inhibited due to a PNPase knockout in *Y. enterocolitica*, the cells restart growth much later after a cold-shock than wild type cells (Neuhaus, Rapposch et al. 2000; Yamanaka and Inouye 2001). Taken together, the *cspA* mRNA or its 5´ end can inhibit cell growth (see also Xia, Etchegaray et al. 2001). We propose here that the CSC-box of *Y. enterocolitica* is a major “signature switch” to regulate the rapid and specific decay of *cspA* mRNA, thus enabling the cells to overcome cold-shock state and enter the low temperature growth phase.

4. 4 Which RNase is responsible for cleavage of the CSC-box?

Due to its central role in the turnover of mRNA, RNase E is a good candidate for the enzymatic activity responsible for highly specific fragmentation of *cspA1/A2* mRNA under cold shock. The widely accepted view is that this enzyme specifically cleaves in A/U-rich single stranded regions that are usually preceded or followed by stable stem-loop structures. Although the exact mechanism of how RNase E select sites for cleavage remains unknown (Coburn and Mackie 1999), the composition of the CSC-boxes and their local structure (computer simulations, data not shown) suggest that they could be very good targets for the endonucleolytic activity of this enzyme. We have shown that at least one of the short RNA oligomers containing the extended CSC-box sequence and the AGUAAA motif from the “cut 2” site was recognized and cleaved by the catalytic domain of RNase E (16mer-cut2). However, the fact that RNase E did not cut the “cut 3” RNA oligomer (16mer-cut3) lacking the extended CSC-box sequence, suggests that the activity of this enzyme is not only dependent on the core AGUAAA sequence, but also on the adjacent regions (Fig. 3.5).

In other organisms RNase E cut sites within cold-inducible genes also have been proposed. (O'Connell, Gustafson et al. 2000) found that in *Sinorhizobium meliloti cspA* has been organized in an operon, but unlike *Y. enterocolitica* (Neuhaus, Francis et al. 1999), *cspA* was combined with non-homologues genes. Preliminary results from (O'Connell and Thomashow 2000) show that approximately 1h after cold shock, a smaller mRNA species appears. Based on theoretical considerations, these authors speculated that this could be due to two putative RNase E cut sites, AUAUU and CGCAUAAA. Also, in *E. coli*, the extreme temperature instability of *cspA* mRNA was found to be partially due to degradation by RNase E at the putative cutting site AAUUAUUA (Fang, Jiang et al. 1997). It is likely that cells control the rate, and also the specificity, of the mRNA decay by RNase E for regulatory purposes (Coburn and Mackie 1999; Nicholson 1999; Steege 2000). Unfortunately, how cells regulate mRNA decay in a time or temperature dependent manner is not fully understood. Cleavage of the artificial mRNA fusion pD5, consisting of the changed CSC-box AGUCCC within *lacZ*, increased shortly (2 min) after cold-shock and than at later time points the decay

returned to pre-shock level (Fig. 3.2), while the modified *lacZ* transcript was present all the time. A possible mechanism to obtain such a fast adaptation in the cutting velocity may be folding of the particular mRNA, as was found for *cspA* mRNA in *E. coli* (Fang, Jiang et al. 1997).

To further prove that RNase E is responsible for cleavage and dependent on flanking sequences, total mRNA from *Y. enterocolitica* was *in vitro* analyzed with RNase E and RNase G enzymes (Fig. 3.6). Results from *in vitro* experiments with total mRNA from *Y. enterocolitica* and with specific oligoribonucleotides for “cut2” and “cut3” proved that degradation of mRNA in the region of specific CSC box is at least partially dependent on RNase E and not dependet on RNase G at all. These experiments were performed with RNase enzymes purified from *E. coli*. It can be proposed that RNase E from *Y. enterocolitica* is highly specific and is responsible for specific cutting, or more likely that some additional factor(s) in the degradosome complex contribute to precise cutting within the CSC box directing RNase E to specific site(s) only in cold.

In the last ten years, significant advances have contributed to the knowledge of mRNA decay and results revealed unexpected similarities between mechanisms of mRNA decay in the pro- and eukaryotes (Regnier and Arraiano 2000; Steege 2000; Yamanaka and Inouye 2001; Huttenhofer, Brosius et al. 2002). For instance, poly(A) tails have a role in the control of mRNA decay in both systems (Ross 1996; Feng, Huang et al. 2001). Other similarities include the physical association of proteins that cooperate in the decay process in multiprotein complexes and possibly the role of 5’ end modification as the first step in degradation in both systems (Rauhut and Klug 1999). Much work is still required, however, to fully understand this important aspect of gene expression. In bacteria, mRNA can be degraded by a number of parallel mechanisms acting independently on different target sites (Regnier and Arraiano 2000). The rate of degradation is dominated by the fastest rate-determining step. Rates of degradation depend on the targets to specific ribonucleases present in the cell. The availability of targets for RNase E, RNase III, RNase G and PNPase depends on several factors, such as RNA folding, protection by ribosomes, and polyadenylation, which modulate mRNA stability. The lack of homology between sequences flanking the sites processed by these RNases highlights the importance of secondary structures in the process of RNA recognition and cleavage by ribonucleases (Regnier and Arraiano 2000). Such a mode of

recognition is presumably linked to the fact that a handful of ribonucleases are able to degrade the thousands of different mRNAs present in the cell. Most of the ribonucleases involved in mRNA metabolism are also implicated in the processing of rRNAs (Bessarab, Kaberdin et al. 1998). Utilization of a limited number of ribonucleases to ensure all RNA cleavages in the cell presumably saves the energy that would be required to synthesize a larger set of specific ribonucleases. However, this might not be the only reason why there is an indirect connection between the synthesis of the translation machinery and RNA decay. The augmentation of ribosomes that accompanies acceleration in growth rate requires an increased synthesis of ribosomal components. This may, in part, be fulfilled by stabilization of the respective mRNAs, that cannot be degraded by ribonucleases engaged in the processing of newly synthesized rRNA precursors (Lopez, Marchand et al. 1998). Moreover, protection of mRNA by translating ribosomes may also contribute to the increase in ribosomal protein synthesis observed upon acceleration of growth. Changes in environmental conditions also modify mRNA stability (Vytvytska, Jakobsen et al. 1998; Repoila and Gottesman 2001) although what detects these changes and how the signal is transmitted to the degradation machinery is not understood. Future work will involve the identification and study of the mechanism of action of additional RNases, relating these RNases to mRNA decay through the isolation of mutants, and assessing how the various reactions are regulated.

Therefore was interesting to go further with characterisation of factors and proteins, which are possibly involved in mRNA degradation process, and assembeling of degradosome complex. One of the most important enzymes for mRNA degradation is PNPase (polynucleotide phosphorylase). It has been shown that PNPase is also cold-shock inducible (Goverde, Huis in't Veld et al. 1998), but complete characterization of the genes adjacent to the PNPase in *Y. enterocolitica* has not been reported in literature yet.

4. 5 Significance of Nlpl protein for cold shock

In this work we have presented the evidence that *pnp*, *nlp1* and *deaD* are part of polycistronic operon and they are cotranscribed as cold temperature induced mRNA. Two transcripts, a large one (5,4kb) encoding *pnp*, *nlp1* and *deaD* genes,

and a smaller one (2,7kb) encoding *deaD* gene with long 5' UTR region, were detected in *Y. enterocolitica* at 10°C (Fig. 3.9). This means that all three genes are cotranscribed on cold temperature and therefore the regulation of the synthesis of all three proteins can be coordinated. If one assumes that *pnp* and *deaD* are described like cold-shock inducible genes, than the *nlp1* gene should be designated as cold-shock inducible as well, due to its presence in the polycistronic mRNA.

NlpI protein was not detected in cell envelopes obtained from wild-type cells of *E. coli* K12, that were grown at 37°C (Ohara, Wu et al. 1999). The function of NlpI is not known yet, but according to the first characterization from *E. coli* this lipoprotein is involved in cell division process (Ohara, Wu et al. 1999), which can be very important in restarting cell growth at low temperatures. Local amino acid sequence similarities suggested an additional role for NlpI protein. On N-terminus of NlpI, TPR (tetratricopeptide repeat) domains containing three tandem TPR motifs were found. The TPR domain is responsible for activation and 25-fold stimulation of protein phosphatase (PP5) activity in yeast, by polyunsaturated fatty acids such as arachidonic acid (Das, Cohen et al. 1998). Most TPR containing proteins are associated with multiprotein complexes, and TPR motifs are important for the functioning of chaperones, cell-cycle, transcription, and protein transport complexes (Blatch and Lassle 1999). In view of the essential role of TPR domains in protein-protein interaction, further studies of NlpI protein and its TPR domain will shed new light on involvement of NlpI protein in cold-shock adaptation.

4. 6 Regulation of *deaD* gene expression during a cold shock

The cold-shock gene expression of *deaD* and its adjacent genes may be regulated by a number of elements in both transcriptional and posttranscriptional level. On the transcriptional level *deaD* contains both an AT-rich upstream element that functions as a transcriptional activator and a cold shock-like box (Fig. 3.7) which is involved in transcriptional attenuation of *cspA* in *E. coli* (Jiang, Fang et al. 1996). Further more, *deaD* may be negatively regulated by its relatively long, highly structured 5' UTR (231 nucleotides), similar to that observed in other cold-shock induced genes (Jiang, Fang et al. 1996; Neuhaus, Francis et al. 1999). Results from this study clearly demonstrate the complex regulation of DeaD synthesis at

both transcriptional and translational level. High mRNA stability was proved by experimental analyses and has been highly connected with specific mRNA degradation during a cold shock. Note that mapping of the 5' end for *pnp* gene was completely in correlation with downstream RNase III cleavage site previously determined in *E. coli* (Jarrige, Mathy et al. 2001). A long irregular stem-loop is predicted to form in the *pnp* leader region by *mfold* program (Mathews, Sabina et al. 1999) and this region is highly conserved in *E. coli*, *Y. enterocolitica* and *Photorhabdus* sp.

Potential secondary structure for RNase III processing was not found in front of *deaD* gene and it was assumed that the detected primer extension product reveals transcriptional start site for *deaD* mRNA synthesis. The other detected primer extension product within the *nlp1* gene was in specific stem-loop structure predicted by *mfold* program (Mathews, Sabina et al. 1999) and pointed one more possible site for RNase III processing. Presence of specific terminator structures at the end of *pnp* gene and at the end of *deaD* gene (Fig. 3.8) is also imoprtant for specific mRNA stabilization and detection during a cold shock. It has been shown over Northern blot analyses that termination at the end of the *pnp* gene during a cold-shock is reduced and the band (5,4kb in length) is stable and detectable under this condition. According to this data it was assumed that *deaD* gene is transcribed from P*pnp* promoter and from its own promoter during a cold shock.

4. 7 EGFP reporter system is a good choice for analysis of cold-shock response

To establish if the induced expression of *deaD* gene on cold was due to cold inducible promoter or due to mRNA stabilization, we decided to place different regions of the *pnp-nlp1-deaD* locus of *Y. enterocolitica* in front of the *egfp* reporter gene. However, as cold-shock may severely alter both stability and translatability of mRNAs, it is particularly important to choose a reporter gene whose expression is minimally affected by such concurrent phenomena. It was shown that synthesis of the GFP protein is very stable in different organisms, at wide ranges of temperatures (Li, Zhao et al. 1998), and is a good alternative to β-galactosidase often used for quantitative characterizations (Scholz, Thiel et al. 2000). This is the first report of using EGFP system for transcriptional analysis during cold-shock

conditions. The other reporter genes (β-galactosidase and CAT – chloramphenicol acetyltransferase) were used in previous studies to show the role of *cspA* promoter sequences and adaptation of translational apparatus in cold-shock response (Goldenberg, D., Azar et al. 1997). Efficiency of translation of *cspA*, *lacZ* and *cat* mRNA was determined during cold shock. It was shown that the cell's protein biosynthetic apparatus is very efficient at translating the cold – shock *cspA* mRNA, and rather inefficient in translating "normal" mRNA, such as *cat* or *lacZ* mRNA. As the *lag* phase caused by the cold-shock comes to its ends and the cells start growing again, this situation begins to reverse and translation of "normal" mRNA becomes efficient with a substantial time delay (Goldenberg, D., Azar et al. 1997). In this work the level of EGFP product expressed from different transcriptional fusions, increases and accumulates with a substantial time delay, also. This delay and the lower increase of the protein level at low temperature (10°C) are not typical of the cold-shock proteins (e.g. CspA, CsdA, Rbf, H-NS). They are synthesized with a much shorter delay due to the efficient utilization of cold-shock mRNAs by the cold-shock modified translational apparatus (Goldenberg, D., Azar et al. 1997); (Brandi, Pietroni et al. 1996). There is no perfect reporter gene to study cold-shock response, but if it is assumed that the transcripts of the same reporter gene display the same degradation rate in a given intracellular milieu, than the difference in the level of measured EGFP product should reflect primarily differences in transcriptional activity. The activity levels of the reporter gene, on the other hand, should reflect the level of corresponding transcripts from different promoters analyzed under the same conditions.

4. 8 Characterization of promoter activity on cold is imoprtant for expression of recombinant proteins

The high-level expression of both heterologous and host proteins in *E. coli* is often accompanied by the formation of inclusion bodies, which are irreversible aggregates consisting mainly of the overproduced polypeptide (Bowden and Georgiou 1990). Inclusion body proteins can generally be refolded in an active conformation. However, recovery yields are highly variable, and unfolding and refolding conditions must be optimized on a case-by-case basis (Bowden and Georgiou 1990). Recombinant protein expression at reduced growth temperatures

presents the combined advantages of increasing the solubility of aggregation-prone recombinant proteins (Schein 1991) and limiting their degradation by heat shock proteases that are induced under overexpression condition. Nevertheless, a sudden decrease in the growth temperature has severe consequences for *E. coli* physiology, including a decrease in the saturation of fatty acids and the inhibition of DNA, RNA, and protein synthesis (Thieringer, Jones et al. 1998; Phadtare, Alsina et al. 1999). More importantly, the efficiency of traditional promoters (e.g., *tac*) is strongly reduced under these conditions (unpublished data from Vasina and Baneyx 1996) and data from this study clearly demonstrate reduction of P*lacZ* promoter activity at low temperature. Since a subset of *E. coli* proteins is actually induced following a temperature downshift the potential of cold shock-inducible promoters in directing the expression of recombinant proteins at low temperatures is very important.

The expression of the transcriptional fusions at 30°C indicated the presence of the promoter activity for the *pnp-egfp* fusion and *deaD2-egfp* fusion, but not for *deaD1-egfp* fusion. The activities of P*pnp* and P*deaD2* promoters were significantly lower than the activity of the *lacZ* promoter at 30°C, when the cells reached early stationary phase. Analyses of EGFP expression after cold-shock revealed that expression of EGFP from P*deaD2* promoter was cold-shock induced and EGFP was expressed at 10°C up to 20h incubation on cold. Results also suggest that efficiency of the traditional P*lacZ* promoter has been strongly reduced under cold shock. Moreover P*deaD2* promoter is active at 30°C and at 10°C, what can be very useful for possible expression of recombinant proteins at low temperatures. Therefore, further analysis of stability in expression of different recombinant proteins from *deaD2* promoter should be pursued.

4.9 Importance of DeaD RNA helicase for cold-shock adaptation

Data from this work suggest that *deaD* gene is transcribed from *pnp* promoter and from its own promoter during a cold shock. Transcription from *pnp* promoter is constitutive, and from *deaD2* promoter is cold-shock inducible. Stabilization of mRNA is involved in synthesis of DeaD protein, due to accumulation of polycistronic transcript during a cold shock, which is not dependent on cold

inducible *pnp* promoter. This means that both transcriptional and posttranscriptional controls are involved in regulation of *deaD* gene expression. More extensive study of the function of DeaD (CsdA) proteins during cold-shock adaptation is necessary. Non-conserved C-terminal region of DEAD proteins has RNA binding specificity and plays a role in forming protein complexes (Liou, Chang et al. 2002). High homology in this region between DeaD and DbpA proteins was considered most likely to be responsible for the RNA specificity whose ATPase activity depends upon the presence of precisely positioned substrate RNA helices (Nicol and Fuller-Pace 1995; Diges and Uhlenbeck 2001).

RNA-binding proteins, in general, are considered to have evolved from a small number of ancient modules (Burd and Dreyfuss 1994; Siomi and Dreyfuss 1997). The RNA binding domains, the S1 domain (S1-D) and the cold-shock domain (CSD) are prime examples of ancient modules that independently can bind RNA. In fact, the CSD is the most conserved nucleic acid-binding sequence described, showing 40 % identity and 60 % similarity between bacteria and vertebrates (Graumann, P. L. and Marahiel 1998). Variations seen in the amino acid sequence of the S1 domain and the CSD might equally well specify different RNA sequence or structure recognition. Similarly, the CSD proteins in eukaryotic cells are found in combination with several other types of module that are thought to confer either a more specific template recognition or an ancillary enzymatic function. In a variety of situations, S1-D or CSD proteins are found in association with DEAD-box RNA helicases, and the two types of protein appear to function together to maintain regions of ssRNA (Blum, Py et al. 1997; Rauhut and Klug 1999; Liou, Chang et al. 2002). CSD proteins are commonly found bound to stored (nontranslating) mRNA, particularly during early growth phase and development (Brandi, Spurio et al. 1999; Regnier and Arraiano 2000). Although complete removal of the CSD proteins from mRNA permits its translation *in vitro*, low concentrations of CSD (alias Csp) protein on the mRNA may be required for maximal translation efficiency *in vivo*. Another component of stored mRNP particles is the protein kinase, which phosphorylates the associated CSD proteins. The loading of CSD proteins on mRNA and the stability of the protein/mRNA complex are regulated by RNA helicase activity and protein phosphorylation.

The ability of CSD proteins to interact with both specific gene promoter elements and nascent transcripts, and to remain associated with mRNA products

destined for initial translational repression but later activation, may mark out a complete pathway for expression of a certain class of genes. How this pathway is established and regulated will require identification of many other components, a few of which have been highlighted here.

5 GENERAL CONCLUSIONS AND FURTHER PROSPECTS

In this work CspA1/A2 and DEAD box RNA helicase were characterized and discussed as proteins with RNA chaperone function. The major weakness of the RNA chaperone hypothesis is that however, no protein had ever been shown to act as an RNA chaperone *in vivo* (Lorsch 2002). According to different studies in last years can be concluded that function of the RNA chaperone is not connected with only one protein, but most probably with specific RNA protein complexes (RNPs) (Graumann, P., Wendrich et al. 1997; Jiang, Hou et al. 1997; Lorsch 2002). The RNA chaperone activity than depends mostly on interaction between different proteins. The analogy was made even more striking upon discovery of the DEAD box class of RNA dependent ATPases (Diges and Uhlenbeck 2001; Mourelatos, Dostie et al. 2002). The RNA binding proteins are divided in two main groups: the RNA specific and the RNA non-specific binding proteins. There are still many important questions to be answered: what are the functions of non-specific RNA binding proteins? Further, do specific RNA binding proteins simply unwind RNA structures and than release ssRNA into solution or is something more complicated going on?

More extensive study of the function of DeaD and NlpI proteins during cold-shock adaptation is necessary. DEAD proteins are thought to be involved in the local unwinding of RNA secondary structures, but newest data suggest a role for DEAD box proteins as RNPases that use chemical energy to remodel the interactions of RNA and proteins (Schwer 2001). In this scenario, DeaD would perform a distinct role in the acclimation of *Yersinia* to the cold-shock state. This role could involve an interaction of the ribosome with the RNA helicases, and/or accessory proteins required for cold acclimation. Specific function of RNA helicases could be achieved over a C-terminus interaction with cold-shock induced and specific RNA substrates to remove cold-stabilized secondary structures in cold-shock mRNAs and remodel interaction of cold-shock mRNAs with ribosome, thereby overcoming the induced blockage of translation initiation under cold shock.

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7 APPENDIX

Following Genbank entries can also be retrieved from the National Center for Biotechnology Information (NCBI) after October 2003 under Numbers:

AF542976 (*pnp-nlpI-deaD* locus) and

AF542975 (RNase III complete cds)

```
====================> BankIt491415            AF542976 (pnp-nlpI-deaD locus)

Submission 2 of a total of 2 submission(s).


LOCUS       bankit491415            6253 bp    DNA     linear   BCT 03-SEP-2002
DEFINITION  Y. enterocolitica pnp, nlp1, dead genes.
ACCESSION   ;
KEYWORDS    .
SOURCE      Yersinia enterocolitica.
  ORGANISM  Yersinia enterocolitica
            Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae;
            Yersinia.
REFERENCE   1  (bases 1 to 6253)
  AUTHORS   Goverde,L., Huis in't Veld,H., Kusters,G. and Mooi,R.
  TITLE     The psychrotrophic bacterium Yersinia enterocolitica requires
            expression of pnp, the gene for polynucleotide phosphorylase, for
            growth at low temperature (5 degrees C)
  JOURNAL   Molecular Microbiology 28(3), 555-569 (1998)
REFERENCE   2  (bases 1 to 6253)
  AUTHORS   Anastasov,N. and Scherer,S.
  TITLE     Identification of cold shock inducible dead gene for RNA helicase
            in Yersinia enterocolitica
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 6253)
  AUTHORS   Anastasov,N. and Scherer,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-SEP-2002) Department of Biosciences, Technische
            Universitaet Muenchen, Weihenstephaner Berg 3, Freising D-85354,
            GERMANY
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====================> bankit487630            AF542975 (RNase III locus)

Submission 1 of a total of 2 submission(s).

LOCUS       bankit487630            2562 bp    DNA     linear   BCT 03-SEP-2002
DEFINITION  RIBONUCLEASE III (RNase III) of Yersinia enterocolitica.
ACCESSION   ;
KEYWORDS    .
SOURCE      Yersinia enterocolitica.
  ORGANISM  Yersinia enterocolitica
            Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae;
            Yersinia.
REFERENCE   1  (bases 1 to 2562)
  AUTHORS   Anastasov,N. and Scherer,S.
  TITLE     Genetic analysis of the rnc operon of Yersinia enterocolitica
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2562)
  AUTHORS   Anastasov,N. and Scherer,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-SEP-2002) Department of Biosciences, Technische
            Universitaet Muenchen, Weihenstephaner Berg 3, Freising D-85354,
            GERMANY
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                     sequences from secreted and periplasmic proteins
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                     PROCESSING OF RIBOSOMAL RNA PRECURSORS AND OF SOME mRNAs.
                       [CATALYTIC ACTIVITY] ENDONUCLEOLYTIC CLEAVAGE TO
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                     ESSENTIAL FOR CELL GROWTH.  [SUBCELLULAR LOCATION]
                     MEMBRANE-BOUND.  [PTM] AUTOPHOSPHORYLATED.  [SIMILARITY]
                     BELONGS TO THE ERA/TRME FAMILY OF GTP-BINDING PROTEINS.
                     ERA SUBFAMILY.  [SIMILARITY] CONTAINS 1 KH DOMAIN"
                     /codon_start=1
                     /product="GTP-BINDING PROTEIN ERA"

/translation="MSEVEKTYCGFIAIVGRPNVGKSTLLNELLGQKISITSRKPQTT

RHRIMGIHTEGPYQAIYVDTPGLHIEEKRAINRLMNRAASSSIGDVELVIFVVEGTNW

TADDEMVVNKLRSLQCPVLLAINKVDNVTDKTKLLPHIQFLSQQMNFLDVVPISAEKG

MNVDTIASIVRKHMPEA"

BASE COUNT      669 a    543 c    661 g    689 t

ORIGIN  1 aaacagaaag acggtaaaaa acgtatgaag caggtcggta acgttgaatt accgcaagag
       61 gctttcctgg ctattctgca cgtcggaaaa gacagtaaat aaggagtttt gatggctaac
      121 atgtttgctt tgattttagc aatagcaacg ctagtgacag ggattatctg gtgcttcgag
      181 cgattcaaat gggggccggc ccgtcaggca aaaattgcgg ctgttaatgc gcaaactgcg
      241 gctatcaagg cacaaaccgg gagtgcggtt gataacaaga ctctggcacc ggcggcaaaa
      301 cagcccggct ggatagagac ttgtgcttca gtcttcccgg tgttagctct ggtatttatc
      361 gtgcgttcgt ttatttacga acccttccag attccttctg gttcgatgat gcccacgttg
      421 ttgatcggtg atttcattct ggtagagaaa tttgcttacg gcattaaaga cccaattact
      481 caaaccacgc tgattcctac cgggcatccc aaacgcggtg atattgccgt gtttaaatac
      541 ccgttagacc ctcgtttgga ctatatcaag cgcgtggtgg gtttaccggg cgaccgcgta
      601 agttataatc cgatcagtaa agaagtaacg gttcagccag catgtaatac cggcgcttct
      661 tgtgatagcg cattgccgat tacttacagt gcatctgagc ctagcgattt cgtgcaaaca
      721 ttccgttata gcggcaatgg tgaggcttct gccggtttct tccaaattcc gctaaatcag
      781 gctgtacctg atggtggtgt gcgtttgaga gagcgtaccg aaaccctcgg cccggttgca
      841 caccagattt tgaccgttcc tgggcgccag gagcagctag gtgcttatta tcaacaacct
      901 aatcagccat taggtgtatg ggttgttcca gaaggtcatt actttatgat gggtgataac
      961 cgggataata gcgcagatag ccgttactgg ggttttgtgc cagaacgtaa tctggttggc
     1021 aaagccacgg ctatttggat gagttttgaa aagcaagaag gtgaatggcc aacgggtgtg
     1081 cgtttaagtc gtattggtgg aatacattaa tactggctct accttgagtc ataggggtga
     1141 cagcggcact gaataccgct gtcacttcaa gcccgcaggg tatttaaacg cagcattata
     1201 tttccactcg ttgtagaata acctttcgag cgataaaagt tggctctttt atgggagcca
     1261 ctgcaaacga aacagttttg tcttggtatt attggccgat cagacctgtt ccgtctgctg
     1321 tagtttttga cgcattcttg atctattggt aactcatgaa ccccatcgta ataaataggc
```

```
1381 tgcaacggaa gctgggctac acttttcaac agcaggatct cttgctgcaa gcactgactc
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1501 gtttcgttat tgccaatgag ttgtaccgcc gtttccctcg tgttgatgaa ggggatatga
1561 gtcggatgcg agcaaccttg gttagaggta atacgctagc cgaaatggcc cgtgagtttg
1621 acctcggtga atgtttacgc cttgggccgg gtgaattgaa aagtggtggt ttccgccggg
1681 aatcaatttt ggcagataca gtcgaagcgt taatcggcgg tatattctta gacagcgata
1741 ttcatacgat tgaacggctg attctcgcct ggtatcacag tcgattagaa gaaatcagcc
1801 ctggtgataa gcagaaagac ccaaaaacgc gcctgcaaga atatttacag ggccgtcatc
1861 tgccattacc ttcttatctg gttgtgcagg ttcgtggtga agcacatgat caggaattta
1921 ctatccactg ccaggtgagt ggcttgaatg aaccggtaat aggtaccggc tcaagtcgcc
1981 gtaaagctga gcaggcggca gcggaacaag cgctgaaaca gttggagctt gaatgagcga
2041 agtagaaaaa acgtattgtg gttttattgc gattgtaggt cgcccgaatg tgggtaaatc
2101 taccttgctg aatgaattgc tggggcagaa aatctccatc acttcacgta aaccgcaaac
2161 cacacgtcac cgtattatgg ggatccacac tgaagggcct tatcaggcca tttatgttga
2221 taccccaggg ctacatattg aagaaaaacg tgcgattaac cgcttaatga atcgcgcggc
2281 aagtagctct atcggtgatg tagaattagt tatttttgtg gttgaaggga cgaactggac
2341 tgctgacgat gaaatggtag ttaataagct gcgcagtttg caatgcccgg tattattagc
2401 catcaacaaa gtcgataacg taacagataa aactaagctg ttgcctcata tccagttctt
2461 aagccaacag atgaatttct tggatgttgt tcctatttct gctgaaaaag ggatgaatgt
2521 ggacactatc gccagcatcg tgcgcaagca tatgccggaa gc
```

//

DNA sequence of RhlB helicase (*rhlB* gene) in *Y.eneterocolitica*

```
ccgctaaaca cccagcttaa cccatcatct tcaaagggta ttgacggcta     50
acgccgctta cagattcgca tccaagaatt ctttaagctg acctttagac     100
aaggcaccaa ccttggttgc cacaacttca ccatcacgga ataacaacag     150
cgtagggatg ccacggatac cgtatttggg tgccgtgccc tggttgtcat     200
caatattcaa tttagtgatg gtaagtctgc cttcatactc ttcagcaatt     250
tcatccaaaa tcggagcaat catcttgcac ggaccacacc attcagccca     300
gaaatcgacc aaaaccaagc cgctggcttt cagcacgtca gtgtcgaagc     350
tgtcgtcact caggtgaata attttatcgc tcatgttcta ctccaaagga     400
ttgtgtctac ctcgttggtg tagcattaac caacaatagg atgactttat     450
ttcaccggat acgctttcgt aaagcaatag ttagctgata ttctaccaca     500
ctatgagcaa aacacacttg accgaacaga agttttccga cttcgccctg     550
cacccgctag ttattgaagc ccttgaaaac aaagggtttc agtattgcac     600
gccgatccag gcgttagcat tgccactcac cctctctggg cgtgatgtag     650
cgggtcaggc gcaaacagga accggcaaga cgctggcatt cttagcgtct     700
actttccatt atttgctttc tcaccccgca gaagagggtc gtcagactaa     750
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ttcactccga tgcagagtca ctttcccagg taaccggcct gaaattaggt     850
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tggcgtagat attcttatcg ggactactgg ccgtttaatc gattacgcaa     950
aacaaaatta tataaatctc ggtgcgattc aggttgtggt attggatgag    1000
gctgatcgga tgtacgattt aggctttatt aaagatatcc gctggctgtt    1050
ccgccgtatg ccttccgttg ataagcgttt aaacatgctg ttctctgcca    1100
cactttcgta ccgcgtacgt gaattggctt tcgaacaaat gaacaacgcc    1150
gaatatgttg aagtggaacc gttacaaaaa accggccacc gcattcagga    1200
agagctgttc taccttcaa atgaagaaaa aatgcgtctg cttcagacgt     1250
tgattgaaga agagtggcca gatcgttgca ttatttttgc caataccaaa    1300
catcgttgtg aagaaatctg gggccacctg gctgccgatg gtcatcgtgt    1350
cggtctattg accggtgatg tcgcacagaa aaaacgcctg cggattttag    1400
aagatttcac caaaggtgat ttggatatcc tggttgcaac tgatgttgcg    1450
gctcgtggtt tgcatattcc actggtaacc cacgtcttta actatgatct    1500
gcctgatgat tgtgaagact atgttcaccg cattggccgt accggtcgtg    1550
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aacttaccgg ctattgagac ctataccggc catagcattc cggtgagtaa    1650
atataatagc gatgcactat taactgattt gccggcgcca aaacgtctgg    1700
cccgtacccg caccgggaat ggcccacgtc gtaactctgc cccacgtcgc    1750
agtggtgcac cacggaataa ccgtaagcga ccgagctgat aaatgatgct    1800
aagttccacc tcactttatg ctgccatcga tcttggctcc                1840
```

Protein sequence of RhlB helicase from *Y. enterocolitica*

>RhlBYe, frame+3, 428 aa

```
503   MSKTHLTEQKFSDFALHPLVIEALENKGFQYCTPIQALALPLTLSGRDVA
      GQAQTGTGKTLAFLASTFHYLLSHPAEEGRQTNQPRALIMAPTRELAVQI
      HSDAESLSQVTGLKLGLAYGGDGYDKQLKVLESGVDILIGTTGRLIDYAK
      QNYINLGAIQVVVLDEADRMYDLGFIKDIRWLFRRMPSVDKRLNMLFSAT
      LSYRVRELAFEQMNNAEYVEVEPLQKTGHRIQEELFYPSNEEKMRLLQTL
      IEEEWPDRCIIFANTKHRCEEIWGHLAADGHRVGLLTGDVAQKKRLRILE
      DFTKGDLDILVATDVAARGLHIPLVTHVFNYDLPDDCEDYVHRIGRTGRA
      GESGHSISLACEEYALNLPAIETYTGHSIPVSKYNSDALLTDLPAPKRLA
      RTRTGNGPRRNSAPRRSGAPRNNRKRPS*                    1789
```

DNA sequence of Enolase (*eno* gene) in *Y.eneterocolitica*

```
aacttgtact gaggaaaatc ta**atg**tccaa aattgttaaa gtcatcggtc      50
gtgaaatcat cgactcccgt ggtaatccaa ctgtagaagc cgaagttcat     100
ttagaaggcg gtttcgttgg tttggctgcg gcaccatcag gtgcttctac     150
tggttcccgt gaagcactgg aattgcgtga cggtgacaaa tcccgtttcc     200
tgggcaaagg cgttctgaaa gccgttgcag cagtaaatgg cccaattgct     250
caggcagtta tcggtaaaga tgccaaagat caggctaata tcgataaaat     300
catgatcgat ctggacggta ctgagaacaa atcccagttt ggtgctaacg     350
ccatcttggc tgtttctttg gcagcagcga aagcagcagc agcatctaaa     400
ggcatgcctt tgtatgagca cattgctgaa ctgaatggca ccccaggcaa     450
attctcaatg ccactgccta tgatgaatat catcaatggc ggcgaacatg     500
ctgataacaa cgttgatatt caagagttta tgattcagcc agtcggggct     550
aaaactctga aagaagccgt tcgtatcggt tctgaagtgt tccatacttt     600
ggctaaggtt ctgaaagcta aaggtatgag cactgcggta ggcgacgaag     650
gtggctacgc gccaaacctg ggttctaacg ccgaagcact ggctgttatc     700
gctgaagcgg taaaacaggc tggttacgag ttgggcaaag acgtgactct     750
ggcgatggac tgtgctgcat ctgaattcta taaagatggc aaatatgttc     800
tggctggcga aggcaacaaa gctttcactt ctgaagagtt cactcacttc     850
ctggaagacc tgaccaaaca gtacccaatc gtctctatcg aagacggtct     900
ggacgaatct gactgggctg gcttcaaata ccagaccgaa gttctgggtg     950
acaaaatcca gttggttggt gacgatctgt ttgtaaccaa caccaagatc    1000
ctgaaagaag gtatcgagaa aggtgttgct aactctatcc tgattaaatt    1050
caaccagatc ggttctctga ccgaaactct ggctgcgatt aagatggcaa    1100
aagacgcagg ttacaccgcg gttatctctc accgttcagg tgaaaccgaa    1150
gatgcgacta tcgctgactt ggcagtcggt accgcagcag gccaaatcaa    1200
aaccggttct atgagccgtt ctgaccgtgt tgctaaatac aaccaactga    1250
tccgtattga agaagcgctg ggcgaccgcg caccattcaa cggtctgaaa    1300
gaagttaaag gccag**taa**tt actgcgctct actttcttaa                1339
```

Protein sequence of Enolase from *Y. enterocolitica*

>EnoYe, frame+3, 431 aa

```
23    MSKIVKVIGREIIDSRGNPTVEAEVHLEGGFVGLAAAPSGASTGSREALE
      LRDGDKSRFLGKGVLKAVAAVNGPIAQAVIGKDAKDQANIDKIMIDLDGT
      ENKSQFGANAILAVSLAAAKAAAASKGMPLYEHIAELNGTPGKFSMPLPM
      MNIINGGEHADNNVDIQEFMIQPVGAKTLKEAVRIGSEVFHTLAKVLKAK
      GMSTAVGDEGGYAPNLGSNAEALAVIAEAVKQAGYELGKDVTLAMDCAAS
      EFYKDGKYVLAGEGNKAFTSEEFTHFLEDLTKQYPIVSIEDGLDESDWAG
      FKYQTEVLGDKIQLVGDDLFVTNTKILKEGIEKGVANSILIKFNQIGSLT
      ETLAAIKMAKDAGYTAVISHRSGETEDATIADLAVGTAAGQIKTGSMSRS
      DRVAKYNQLIRIEEALGDRAPFNGLKEVKGQ*                1318
```

The following plasmid maps are presented additionally for possible further usage. All plasmids used in this study have been deposed in Wiehestephan Stamm Samlung Genetic Modified Organisms (WSGMO). Specific numbers are listed in Tab. 2.2.

pBSK(K)1

GMO/Vector collection number: WSGMO 7082

GMO/Vector short description: Plasmid pBlueScriptSK(K)1 in *Escherichia coli* XL1 blue cells

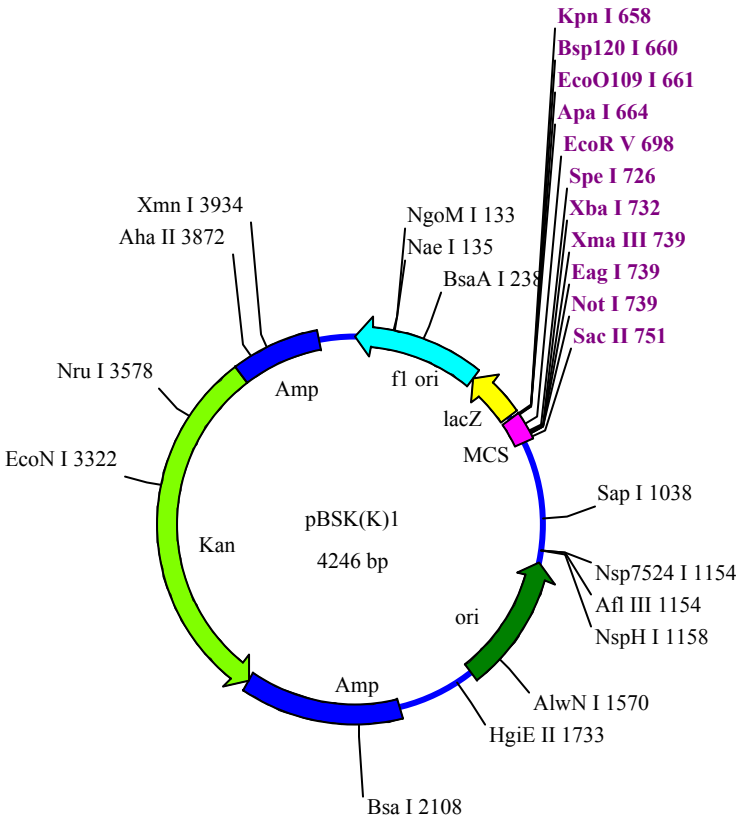
GMO/Vector purchased from: By subcloning an specific Kan^R cassette from pUC4 plasmid in *Sca*I restriction enzyme site in ampicillin gene of pBlueScript SK(-). Construct made for further use in *Y. enterocolitica* strain. (Nataša Anastasov)

References concerning the origin of the GMO/vector: Sambrook J, Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Particular uses of the GMO/vector: Specific plasmid for laboratory purpose. (Nataša Anastasov)

References concerning the particular uses of the GMO/vector: Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidmann, J. G., Smith, J. A. and Struhls, K. 1987. Current protocols in Molecular Biology.

Vector map:



pKCsdA4

GMO/Vector collection number: WSGMO 7079 (Ec_pKCsdA4)

GMO/Vector short description: *Escherichia coli K12 CC118(λpir)* pKCsdA4

GMO/Vector purchased from: pKNG101 vector; Belgian Co-ordinated Collections of Microorganisms (BCCM) Adress: BCCM, Collection of the Laboratorium voor Microbiologie en Microbiele Genetica, Rijksuniversiteit, Ledeganckstraat 35, B-9000, Gent, Belgium.

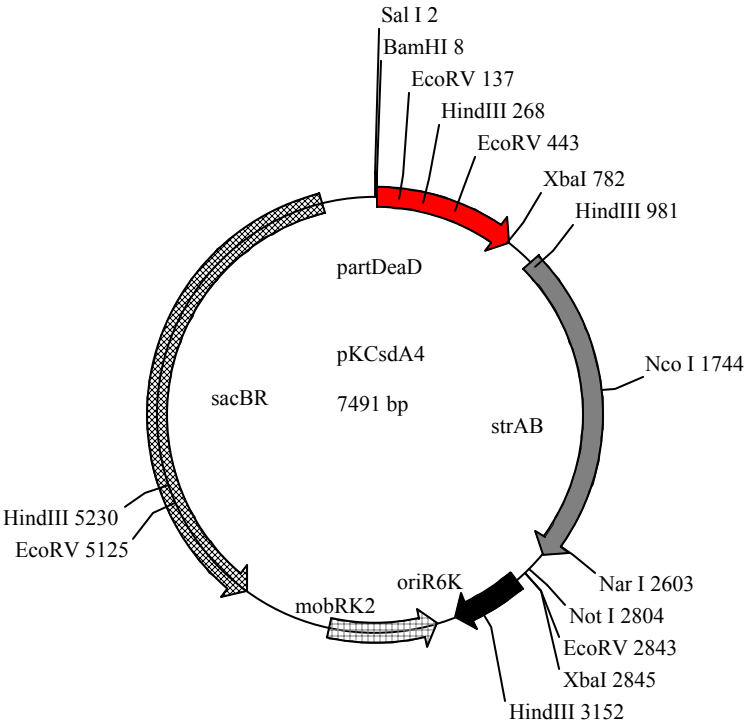
GMO/vector received: pKCsdA4 construct was made in July 2002

References concerning the origin of the GMO/vector: KANIGA, K., I. DELOR, et al. (1991). "A wide-host-range suicide vector for improving reverse genetics in gram- negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*." Gene **109**(1): 137-41.

Particular uses of the GMO/vector: Contains a suicide vector to knockout CsdA (DeaD) gene by double crossover. Vector contains a conter-selection for reducing undesired backround mutants (single crossovers i.e.). Drawback: low copy plasmid pKNG101 (=WSGMO7039). Improved version (high copy) is pMRS101 (=WSGMO 7040)

Remarks from LMBP: pKNG101 is a mobilizable suicide vector that facilitates the positive selection of double recombination events in Gram-negative bacteria. The plasmid contains: (i) the defective pir- origin of replication of the plasmid R6K, (ii) the RK2/RP4 origin of transfer, (iii) the strA and strB genes of RSF1010 encoding streptomycin phosphotransferase as a selection marker for integration, (iv) the sucrose-inducible sacB gene of Bacillus subtilis, encoding levansucrase as a positive selection marker for excision and (v) a multiple cloning site.

Vector map:



pKΔRhlB18

GMO/Vector collection number: (Ec_pKΔRhlB18)

GMO/Vector short description: *Escherichia coli K12 CC118(λpir)* pKΔRhlB18

GMO/Vector purchased from: pKNG101 vector; Belgian Co-ordinated Collections of Microorganisms (BCCM) Adress: BCCM, Collection of the Laboratorium voor Microbiologie en Microbiele Genetica, Rijksuniversiteit, Ledeganckstraat 35, B-9000, Gent, Belgium.

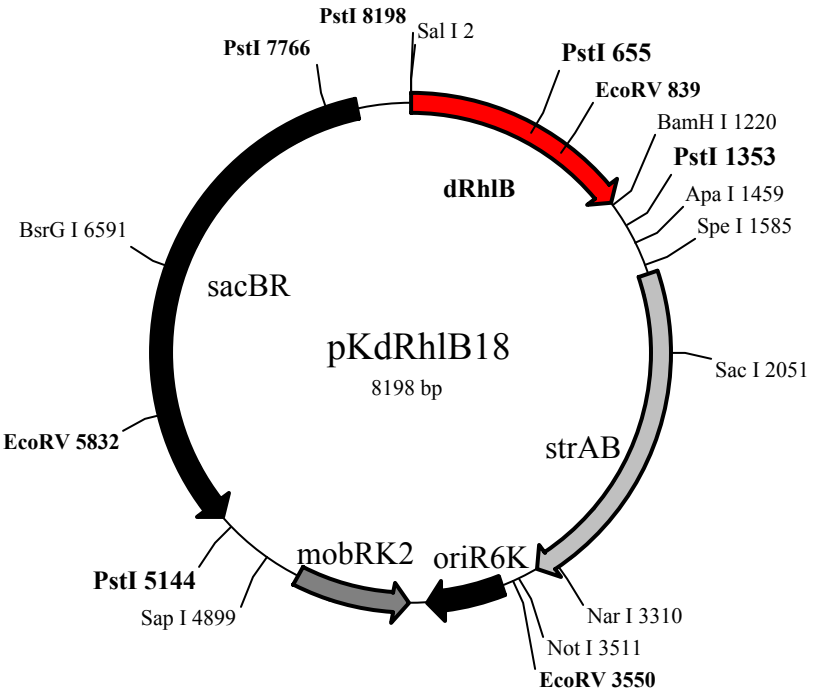
GMO/vector received: pKΔRhlB18 construct was made in July 2002

References concerning the origin of the GMO/vector: KANIGA, K., I. DELOR, et al. (1991). "A wide-host-range suicide vector for improving reverse genetics in gram- negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*." Gene **109**(1): 137-41.

Particular uses of the GMO/vector: Contains a suicide vector to knockout RhlB gene by double crossover.

Remarks from LMBP: pKNG101 is a mobilizable suicide vector that facilitates the positive selection of double recombination events in Gram-negative bacteria. The plasmid contains: (i) the defective pir- origin of replication of the plasmid R6K, (ii) the RK2/RP4 origin of transfer, (iii) the strA and strB genes of RSF1010 encoding streptomycin phosphotransferase as a selection marker for integration, (iv) the sucrose-inducible sacB gene of Bacillus subtilis, encoding levansucrase as a positive selection marker for excision and (v) a fragment of RhlB gene subcloned in multiple cloning site.

Vector map:



pKΔRhlB(K)2

GMO/Vector collection number: (Ec_pKΔRhlB(K)2)

GMO/Vector short description: *Escherichia coli K12 CC118(λpir)* pKΔRhlB(K)2

GMO/Vector purchased from: pKNG101 vector; Belgian Co-ordinated Collections of Microorganisms (BCCM) Adress: BCCM, Collection of the Laboratorium voor Microbiologie en Microbiele Genetica, Rijksuniversiteit, Ledeganckstraat 35, B-9000, Gent, Belgium.

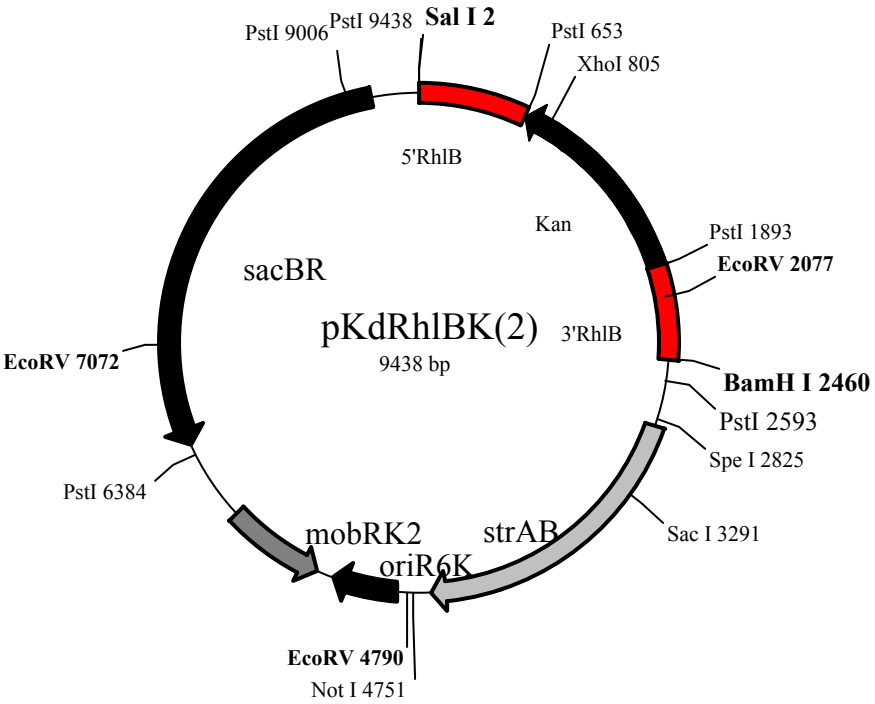
GMO/vector received: pKΔRhlB(K)2 construct was made in July 2002

References concerning the origin of the GMO/vector: KANIGA, K., I. DELOR, et al. (1991). "A wide-host-range suicide vector for improving reverse genetics in gram- negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*." Gene **109**(1): 137-41.

Particular uses of the GMO/vector: Contains a suicide vector to knockout RhlB gene by double crossover.

Remarks from LMBP: pKNG101 is a mobilizable suicide vector that facilitates the positive selection of double recombination events in Gram-negative bacteria. The plasmid contains: (i) the defective pir- origin of replication of the plasmid R6K, (ii) the RK2/RP4 origin of transfer, (iii) the strA and strB genes of RSF1010 encoding streptomycin phosphotransferase as a selection marker for integration, (iv) the sucrose-inducible sacB gene of Bacillus subtilis, encoding levansucrase as a positive selection marker for excision and (v) a fragment of RhlB gene with Km^r casette subcloned in multiple cloning site.

Vector map:



CURRICULUM VITAE

Nataša Anastasov

Personal data:

Date of Birth:	02.12.1971
Place of Birth:	Jagodina, Serbia, Yugoslavia
Nationality:	Serbian (Yugoslavian)
Marital status:	married with Darko Anastasov

Education and Studies:

09/86-06/90	**Secondary School** with Nature-Mathematical education in Jagodina. Final exam Matura (Abitur)**:** Juni 1990
09/90-06/95	**Graduate studies** in Molecular Biology and Physiology, Faculty of Biology, University of Belgrade, Yugoslavia. **B.Sc.** in Molecular Biology and Physiology with Diploma work: Juni 1995
09/95-06/98	**Postgraduate studies** in Molecular Genetics and Genetic Engineering, Faculty of Biology, University of Belgrade. **M.Sc.** in Molecular Genetics and Genetic Engineering: Juni 1998
03/99-06/03	**Ph.D. (Promotion)** at Institute of Microbiology, Department für Biowissenschaftliche Grundlagen, Wissenschaftszentrum Weihenstephan (WZW),Technische Universität München. **Ph.D.** Juni 2003

Working experience:

01/96-03/99	**Research assistant position** at the Institute of Molecular Genetics and Genetic Engineering, Belgrade.
06/98-07/98	**FEMS Young Scientist Grant** for assistance in attending the 8th International Symposium on the Genetics of Industrial Microorganisms, Jerusalem, Israel.
03/99-06/03	Ph.D. research work was supported by **Research grant** from the “Vereinigung der Förderer und Freunde des FML Weihenstephan”.